

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE

7 JUN 95

3. REPORT TYPE AND DATES COVERED

4. TITLE AND SUBTITLE

The Biochemical Remediation of A TNT Contaminated Soil

5. FUNDING NUMBERS

6. AUTHOR(S)

David Gene Young

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

AFIT Students Attending:

Auburn

8. PERFORMING ORGANIZATION REPORT NUMBER

95-031D

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

DEPARTMENT OF THE AIR FORCE

AFIT/CI

2950 P STREET, BLDG 125

WRIGHT-PATTERSON AFB OH 45433-7765

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for Public Release IAW AFR 190-1

Distribution Unlimited

BRIAN D. Gauthier, MSgt, USAF

Chief Administration

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

DTIC QUALITY INSPECTED 1

14. SUBJECT TERMS

15. NUMBER OF PAGES

352

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT

18. SECURITY CLASSIFICATION OF THIS PAGE

19. SECURITY CLASSIFICATION OF ABSTRACT

20. LIMITATION OF ABSTRACT

THE BIOCHEMICAL REMEDIATION OF A TNT CONTAMINATED SOIL

David Gene Young

Accession For	
NTIS CRA&I	<input checked="checked" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
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Distribution /	
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Dist	Avail and/or Special
A-1	

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Style manual of journal used Water Research

Computer software used Word Perfect 6.0a for
Windows

THE BIOCHEMICAL REMEDIATION OF A TNT CONTAMINATED SOIL

David Gene Young

A Dissertation
Submitted to
the Graduate Faculty of
Auburn University
in Partial Fulfillment of the
Requirements for the
Degree of
Doctor of Philosophy

Auburn, Alabama

June 7, 1995

VITA

David Gene Young, son of Cecil and Betty Young, was born April 2, 1953, in LaGrange, Georgia. In 1979, he earned his Bachelor of Science degree in Civil Engineering from Auburn University, Alabama. He worked as an Assistant County Engineer in Morgan County Alabama for two years before joining the United States Air Force where he held several key positions within Air Force Civil Engineering Squadrons at three different assignments. In 1992, he earned the Master of Science degree in Civil Engineering (Environmental) from Auburn University. He began his doctoral studies in Environmental Engineering in the Civil Engineering Department at Auburn University in the Summer of 1992. On March 16, 1972, he married Christie Moncus, daughter of Brunner and Opal (McGill) Moncus. They have two children, Victoria and Jennifer Young.

DISSERTATION ABSTRACT

THE BIOCHEMICAL REMEDIATION OF A TNT CONTAMINATED SOIL

David Gene Young

Doctor of Philosophy, June 7, 1995
(M.S., Civil Engineering, Auburn University 1992)
(B.S., Civil Engineering, Auburn University 1979)

375 Typed Pages

Directed by S. Rod Jenkins

This research presents the first field evidence for the phytoremediation of a TNT contaminated soil by the emerged aquatic plant, *Myriophyllum brasiliense*. Commonly known as Parrotfeather, this plant features a nitroreductase enzyme capable of promoting the reduction of the nitro groups on TNT to the corresponding amino groups. The proposed reductive pathway takes the TNT through isomers of monoamino and diamino to the final triaminonitrotoluene (TAT). Once in the TAT form and in the presence of oxygen, the final oxidative step quickly yields ring opened products and complete phytoremediation of TNT.

Significantly, both TNT and the hazardous amino reduction products were degraded with no buildup observed in

field or laboratory waters when Parrotfeather was present. Another major attribute of the Parrotfeather was that it functioned to degrade aqueous phase TNT in cool weather and under field conditions.

Parrotfeather-induced TNT degradation occurred rapidly once the TNT was solubilized in water with the half-life strongly plant-to-water ratio dependent. At a 1:5 ratio, i.e. grams of Parrotfeather to milliliter of water, the half-life of TNT was 1 hour; whereas, a 1:20 ratio resulted in a half-life of 20 hours. The TNT degradation reaction was described by first-order disappearance kinetics with increasing amounts of plants resulting in larger first-order rate constants.

In the field systems, mass transfer was apparently the rate limiting step. When the experimental setup was favorable for increased mass transfer of TNT from the soil to the water, ten grams of Parrotfeather degraded 35.8 mg of TNT from the soil within the first week, an 82% reduction.

Experimental evidence was also obtained that the presence of Parrotfeather in a TNT contaminated soil and water system promotes bacterial growth in the water and reduces, inhibits and/or eliminates components toxic to several aquatic organisms.

ACKNOWLEDGEMENTS

First, I wish to express my sincere appreciation to my advisor Dr. S. Rod Jenkins for his outstanding guidance and steadfast friendship. The knowledge and practical experience I have gained in my advanced educational endeavor is directly attributable to his superior abilities.

Each member of my committee also provided invaluable assistance and immeasurable support which is gratefully appreciated. Also appreciated was the helpfull information and priceless laboratory assistance provided by: L. Wolfe, L. Carreira, T. Ou, M. Cipollone, J. Entry, K. Flynn, L. Samuelson and G. Wehtje. Special thanks to Mr. Ronnie Wynn, Alabama Army Ammunition Plant site manager, for his patience and many efforts to make the field work function smoothly.

Grateful appreciation is also extended to my wife and children, without who's limitless support and encouragement this would not have been possible.

Finally, the funding support for this study was provided for the most part by the USAF Armstrong Research Lab, Tyndall AFB, Florida and is thankfully acknowledged.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION	1
Background	1
Scope and Objectives	11
Site Assessment Summary	14
Drainage	17
Soil	17
Contamination	19
Methodology	21
Organization	21
 CHAPTER 2: TRINITROTOLUENE (TNT) LITERATURE REVIEW . . .	22
General Information	22
Conventional Military Explosive	22
TNT Properties	23
TNT Manufacture	23
Toxicity	27
Human	29
Animals	32
Other Organisms	33
Mutagen	35

Carcinogen	35
Drinking Water Health Advisory	36
TNT Contamination	36
Air	37
Surface Waters	37
Wastewater	39
Pink Water	39
Red Water	40
Wastewater Handling	41
Groundwater Contamination	43
Soil Contamination	44
DNT Contamination	46
Soil Contamination Migration	47
Temperature Effects	47
Soil Interactions	47
Soil Redox	50
TNT Transformations in Soil	51
TNT Elimination	52
Biological Reduction	52
Aerobic Environment	56
Anaerobic Environment	58
TNT Nitro Group Reduction	60
TNT Photodecomposition	60
TNT Product Photodecomposition	68
Terrestrial Plants	69

Degradation Resistant	70
TNT Contaminant Elimination	72
Incineration	73
Composting	73
Extraction (Surfactant)	77
Biological Systems	79
<i>Phanerochaete chrysosporium</i>	81
<i>Pseudomonas</i>	82
<i>Desulfovibrio</i>	83
<i>Veillonella alkalescens</i>	85
<i>Nitella</i> sp.	85
Analytical Measurements	86
HPLC	88
MECE	89
GC/MS	89
Standard Compound	90
Summary	90
 CHAPTER 3: ANALYTICAL MATERIALS AND METHODS	 93
Chemicals and Standards	93
Analytical Methods	94
High Pressure Liquid Chromatography (HPLC)	95
¹⁴ C Radiochromatography	96
Gas Chromatography/Mass Spectrometry (GC/MS)	96

Micellar Electrokinetic Capillary Electrophoresis	
(MECE)	96
Analytical Procedures	97
HPLC Analysis for TNT	97
HPLC Example Chromatogram	99
¹⁴ C Radiochromatographic Analysis	102
GC/MS	102
MECE Analysis	105
MECE Example Chromatogram	105
UV Spectra of TNT and Reduction Products	108
Field Experiment Aqueous Phase pH Determinations	108
Soil Eh Field Determinations	108
Statistical Determinations	109
General Sample Preparation Procedures	110
Aqueous Phase Sample Preparations	110
Solid Phase Sample Preparations	111
Sample Dilution for HPLC Analysis	111
Microbial Estimation	112
 CHAPTER 4: FIELD EXPERIMENTS	 114
Field Experiment Organization	114
Site selection	114
Site Layout	115
Pond Water Characterization	116
Plant Characterization	119

Parrotfeather	119
Plant Handling	120
Contaminated Soil	121
Soil Site	122
Soil Preparation	123
Soil Field Handling	124
Soil Characterization	125
Field Sampling Methods	131
Aqueous Phase	131
Solid Phase	132
Field Soil TNT Extraction	133
Field Experiment One	134
Experimental Method	135
Experimental Data Analysis	136
TNT Concentration in Treatment Soils	136
TNT Concentration in Treatment Waters	140
Field Water TNT-Product Identification	144
Number of Microorganisms in Soil and Water Field	
Samples	144
Field Observations	145
Problems Identified	146
Field Experiment Two	148
Experimental Method	148
Experimental Data Analysis	149
TNT Concentration in Treatment Waters	150

TNT and Product Identification in Field Water	
.	153
UV Spectral Profiles	156
GC/MS Product Identification	158
Number of Microorganisms in Soil and Water Field	
Samples	160
Nitroreductase Estimation	162
Field Observations	163
Problems Identified	166
Field Water pH Determinations	168
Soil Redox Potentials	171
 CHAPTER 5: LABORATORY EXPERIMENTS	173
Laboratory Materials	173
Laboratory TNT Contaminated Soil	173
Laboratory Plants	174
Laboratory Sampling	175
Aqueous Phase	175
Solid Phase	176
Laboratory Experiments	178
TNT Containing Field Soil - Representative Subsample	
Determination	179
Microbial Analysis in the Presence of TNT	179
Methods and Materials	181
Results and Discussion - Water Phase	184

Results and Discussion - Soil Phase	184
Parrotfeather Effect on Aqueous Phase TNT Concentration	
.	185
Experimental Method	186
Results and Discussion	187
Parrotfeather Effect on Solid Phase TNT Concentration	
.	191
Experimental Method	192
Results and Discussion - Aqueous Phase . . .	195
Results and Discussion - Solid Phase	195
Ten Week Soil-Water TNT Study	200
Experimental Method	200
Results and Discussion - Aqueous Phase . . .	201
Results and Discussion - Solid Phase	204
Aqueous Phase ¹⁴ C-TNT Fate in Parrotfeather and Water	
System	208
Labeled TNT-Water Solution Preparation . . .	208
Experimental Method	209
Analytical Method - ¹⁴ C-TNT Fate in Water . .	211
Results and Discussion - ¹⁴ C-TNT Fate in Water	212
Analytical Method - ¹⁴ C-TNT Fate in Parrotfeather	
.	214
Results and Discussion - ¹⁴ C-TNT Fate in	
Parrotfeather	216
Summary	216

Water Incubated With Parrotfeather Enzymatic TNT	
Reduction Study	223
Experimental Method	223
Results and Discussion	226
Plant, Plant Water, and Axenic Plant Comparison .	226
Experimental Method	227
Results and Discussion	228
Nitroreductase Activity in the Parrotfeather Plant	231
Effect of Plant-to-Water Ratio on TNT Reduction .	231
Experimental Method	233
Results and Discussion	233
Field Water Toxicity	234
Methods and Materials	237
Observations	237
Discussion and Conclusions	239
CHAPTER 6: SUMMARY AND RECOMMENDATIONS	241
Summary	241
Recommendations	243
RERERENCES	246
APPENDICES	
APPENDIX A: TNT EXTRACTION PROCEDURE	271
APPENDIX B: FIELD EXPERIMENT ONE DATA	282

APPENDIX C: FIELD EXPERIMENT TWO DATA	294
APPENDIX D: LAB EXPERIMENTS	304
APPENDIX E: SPECTRAL PROFILES OF TNT AND TRANSFORMATION PRODUCTS	332
APPENDIX F: LISTING OF COMMON ABBREVIATIONS	339
APPENDIX G: LINEAR FIRST ORDER PLOTS	341
APPENDIX H: FIELD EXPERIMENT PHOTOGRAPHS	345

LIST OF TABLES

Table 1.1	Explosives Manufactured at AAAP from 1943 to 1945	16
Table 2.1	Selected TNT Properties	24
Table 2.2	Common TNT Transformation Products, Abbreviations, and a Mode of Generation	28
Table 2.3	Photodegradation Products of an Irradiated TNT Distilled Water Solution [After Burlingson et al. 1979]	63
Table 4.1	Pond Water Sample ICAP Analysis - Taken From the AAAP Beaver Pond Serving as the Field Experiment Water Source	117
Table 4.2	TNT Contaminated Soil Physical and Chemical Characteristics	126
Table 4.3	Results of Metals Analysis of TNT Contaminated Soil From the AAAP Mineral Characteristics	128
Table 4.4	Field Experiment One: Averaged Soil TNT Concentration Sorted by Time and Treatment Method	137
Table 4.5	Field Experiment One: Average Aqueous Phase TNT Concentration Sorted by Time and Treatment Method	142

Table 4.6	Field Experiment Two: Averaged Aqueous Phase TNT Concentration Sorted by Time and Treatment Method	151
Table 4.7	Field Experiment Two: MECE Analysis of the TNT and Reduction Product Concentrations in the Aqueous Phase of the "With Parrotfeather" Treatment . . .	154
Table 4.8	Field Experiment Two: MECE Analysis of the TNT and Reduction Product Concentrations in the Aqueous Phase of the "Without Parrotfeather" Treatment . .	155
Table 4.9	Field Experiment Two: Nitroreductase Enzyme Estimate	164
Table 4.10	Field Experiment Two: Averaged pH Values Sorted by Week and Treatment	169
Table 5.1	TNT Contaminated Field Soil - Representative Subsample Determination	180
Table 5.2	Microbial Analysis in the Presence of TNT - Matrix of Experimental Treatments Investigated . .	183
Table 5.3	Parrotfeather Effect on the Aqueous Phase TNT Concentration Sorted by Time and Treatment Method	188
Table 5.4	Parrotfeather Effect on the Aqueous Phase TNT Concentration in a Beaker With TNT Containing Soil	197
Table 5.5	Parrotfeather Effect on the Solid Phase TNT Concentration - Soil TNT Concentration Remaining in the Experiment Beakers at the End of the Experiment and Sorted by Treatment Method	199

Table 5.6	Ten Week Study - Aqueous Phase TNT Concentration	
	Averaged and Sorted by Treatment	202
Table 5.7	Ten Weeks Soil-Water Study - Averaged TNT	
	Concentration in the Soil Sorted by Time and	
	Treatment	205
Table 5.8	Ten Week Experiment - Total Amount of TNT (mg)	
	Sorted by Treatment by Week	207
Table 5.9	¹⁴ C Activity in Each Major Experimental Component	
	of the TNT-Water System After Incubation With	
	Parrotfeather	218
Table 5.10	Total TNT Concentration in the Aqueous Phase and	
	Extracted Plant-Solvent Solution After Incubation With	
	Parrotfeather	221
Table 5.11	TNT Concentration in the Aqueous Phase Measured	
	Over Time Resulting From Treatments of Water Incubated	
	With Parrotfeather Plants and Parrotfeather Plants	224
Table 5.12	TNT Concentration in the Aqueous Phase Resulting	
	Over Time From Treatments of Parrotfeather Plants,	
	Parrotfeather-Incubated Water, and Axenic Parrotfeather	
	Plants	229
Table 5.13	Aqueous Phase TNT Concentration Resulting From	
	the Varying Ratio of Parrotfeather to Water	235

LIST OF FIGURES

Figure 1.1	U.S. Army Installations With Confirmed and Potential TNT Contamination From the U.S. Army Toxic and Hazardous Materials Agency (USATHAMA) Database [After Tucker et al. 1985]	3
Figure 1.2	Field Experiment, Soil Contamination, and Beaver Pond Sites	13
Figure 1.3	Vicinity Map of the AAAP	15
Figure 1.4	Surface Drainage and Site Overview	18
Figure 1.5	Field Test Range and Contamination Map	20
Figure 2.1	Reaction Sequence for the Formation of 2,4,6- Trinitrotoluene [After Schott and Worthley 1974]	26
Figure 2.2	The Generic Pathway for the Biological Reductive Transformation of a Nitro Aromatic Compound [After Yamashina et al. 1954]	54
Figure 2.3	A Scheme Showing the Formation of Azoxy Compounds Produced by Mild Oxidation of Aromatic Amines [After Smith 1965]	57
Figure 2.4	Proposed Transformation Pathway of TNT by Nitro Groups Reduction [After McCormick et al. 1976]	61

Figure 2.5	Proposed Primary Photo Chemistry Mechanism [After Burlingson et al. 1979]	64
Figure 2.6	Reduction of an Aromatic Nitro Group to the Corresponding Aniline Through the Nitrosobenzene and Phenylhydroxylamine [After March 1985]	66
Figure 2.7	Proposed Biotransformation Pathway for TNT in Compost Systems [After Kaplan and Kaplan 1982d] . . .	76
Figure 2.8	Proposed Anaerobic TNT Transformation Pathway by <i>Desulfovibrio</i> [After Boopathy and Kulpa 1992] . . .	84
Figure 2.9	Proposed Mechanism of TNT Reduction with Extracted Sediment Enzyme and Aquatic Plant Stonewort [After Wolfe et al. 1994]	87
Figure 3.1	A Representative TNT Standards Plot	98
Figure 3.2	Example HPLC Chromatogram for a 1.28 ppm TNT Standard Concentration	100
Figure 3.3	Example HPLC Chromatogram of an Actual Sample Showing Peaks, Retention Times, and Annotations for TNT, MANT, and DAT	101
Figure 3.4	GC/MS Example Chromatogram showing the TNT and Amino Reduction Product Standards	103
Figure 3.5	Example MECE Chromatogram of TNT and the Reduction Product Standards	106

Figure 3.6	Example MECE Chromatogram of an Actual Water Sample Taken From the "With Plants" Treatment in the Second Field Experiment	107
Figure 4.1	Average Soil TNT Concentration By Treatment	138
Figure 4.2	Field Experiment One: Average Aqueous Phase TNT Concentration	143
Figure 4.3	Field Experiment Two Averaged Aqueous Phase TNT Concentration	152
Figure 4.4	MECE Chromatogram of a Field Experiment Two Water Sample Taken at Week 15 From the "With Plants" Treatment.	157
Figure 4.5	MECE Chromatogram of a Field Experiment Two Water Sample Taken at Week 15 From the "Without Plants" Treatment.	157
Figure 4.6	GC/MS Chromatogram of Field Experiment Two Aqueous Phase Parrotfeather Treatment Sample With Identified Reduction Product Peaks Annotated . . .	159
Figure 4.7	GC/MS Chromatogram of Field Experiment Two Aqueous Phase Without Parrotfeather Treatment Sample With Identified Reduction Product Peaks Annotated	161
Figure 4.8	Field Experiment Two Averaged pH Values	170
Figure 5.1	Parrotfeather Effect on the Aqueous Phase TNT Concentration	190

Figure 5.2	Parrotfeather Effect on Kinetics of Aqueous Phase TNT Concentration	193
Figure 5.3	Aqueous Phase TNT Concentration in a Beaker With TNT Containing Soil	198
Figure 5.4	Ten Week Experiment Averaged Aqueous Phase TNT Concentration	203
Figure 5.5	Ten Week Experiment Averaged Soil TNT Concentration	206
Figure 5.6	Comparison Plots of Aqueous Solution Activity, Hours 3 and 73.	213
Figure 5.7	GC/MS Chromatogram of the Aqueous Phase Fate of Radiolabeled TNT Showing the Major Reduction Products After Incubation With Parrotfeather For 73 Hours	215
Figure 5.8	Comparison Plots of Extracted Plant Solution Activity, 3 and 73 hours.	217
Figure 5.9	¹⁴ C Activity in Each Major Experimental Component of the TNT-Water System After Incubation With Parrotfeather	219
Figure 5.10	Total TNT Concentration After Incubation With Parrotfeather	222
Figure 5.11	TNT Concentration in the Aqueous Phase Resulting Over Time From Treatments of Parrotfeather Plants, Parrotfeather-Incubated Water, and Axenic Parrotfeather Plants	230

Figure 5.12 Nitroreductase in Parrotfeather - Double	
Antibody Assay	232
Figure 5.13 Aqueous Phase TNT Concentration Due to Varying	
Plant-to-Water Ratio	236
Figure 5.14 Calculated Least Squares Best Fit Lines for	
Varying Ratio of Parrotfeather-to-Water	238

CHAPTER 1

INTRODUCTION

Background

There are now over 32,000 identified Environmental Protection Agency (EPA) Superfund hazardous waste sites (Abelson 1992). Current cost estimates for the remediation of these sites is over a trillion dollars. Even with the expenditure of this exorbitant amount only a few of them will be restored to a 'pristine' condition (Abelson 1992).

Many of these sites fall under the responsibility of the Department of Defense (DoD) and are contaminated with hazardous energetic nitro-organic compounds (Walker and Kaplan 1992). Although not all are afflicted with energetic pollutants, the DoD has an estimated 12,000 sites that require some form of remediation related to munitions production (Zappi 1994). In the United States alone, beyond the cleanup of other contaminated media, the physical treatment of just the munitions-contaminated soils will cost more than \$1.5 billion (Funk et al. 1993). Additionally, munitions related contamination is an international problem that has not been fully assessed. There are many documented

sites having heavy soil pollution throughout much of Europe and there are probably many additional undocumented sites that will require cleanup (Preuss and Haas 1987).

In the United States (U.S.), trinitrotoluene (TNT) is the predominant conventional bursting charge explosive material selected for military use by the Army early in this century. Since then, TNT was used by all branches of the military and in a variety of munitions (Nay et al. 1974). During World War II, five Army Ammunition Plants (AAPs) manufactured TNT; another 25 load, assemble, and pack (LAP) plants were operated to place the explosives into shells and bombs (Pennington 1988). Accordingly, TNT is one predominate nitro-organic pollutant found at munitions contaminated sites. A map of the continental U.S. showing Army installations with confirmed and potential TNT contamination is included as Figure 1.1.

Primarily, the sites plagued with TNT contamination are at locations where: TNT was manufactured, explosive munitions were constructed and packaged, or TNT-bearing waste materials were discarded. Most of the significant and chronic contamination problems stem from the land application of waste products such as wastewater sludge's,

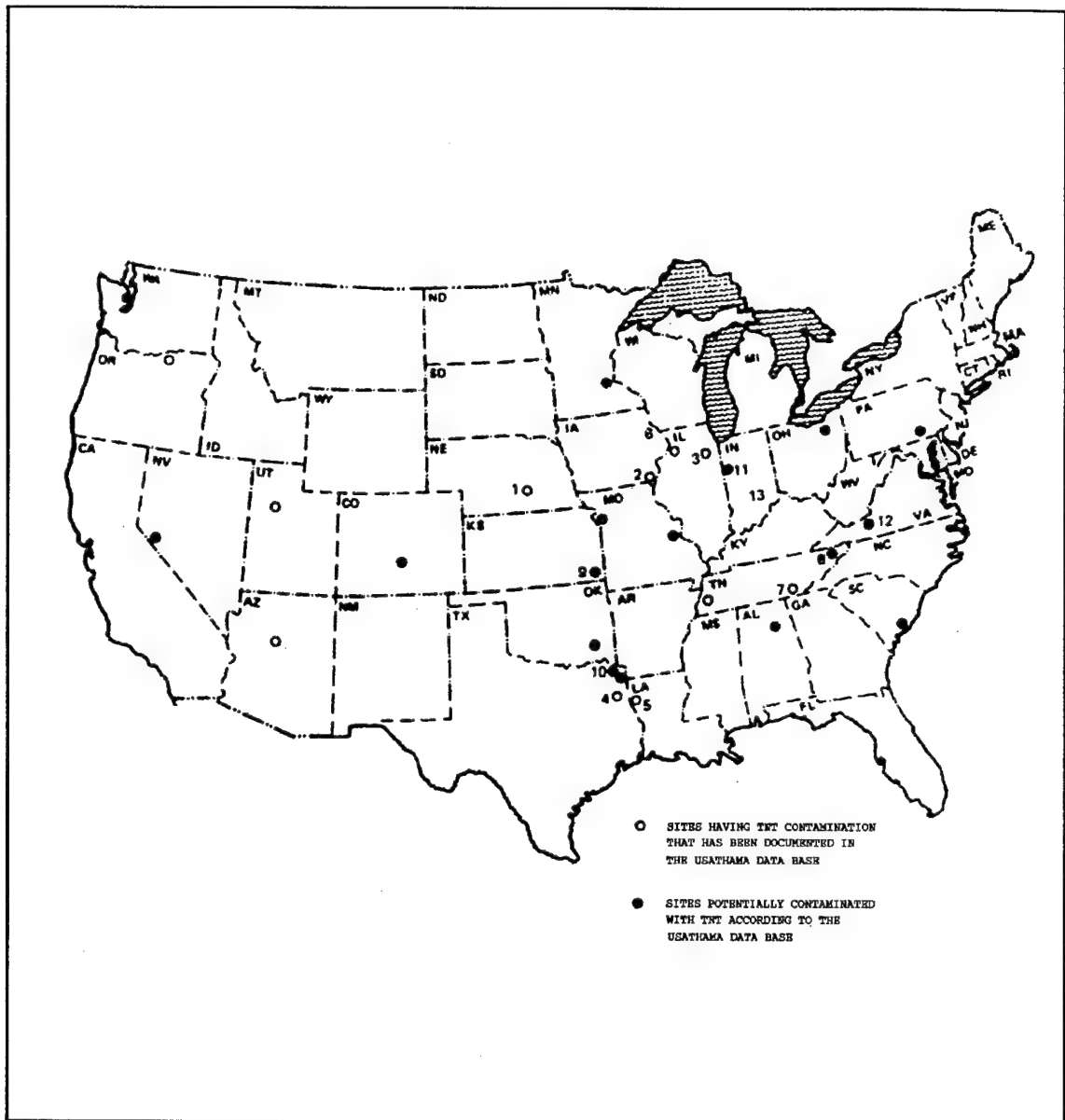


Figure 1.1
U.S. Army Installations With Confirmed and Potential TNT
Contamination From the U.S. Army Toxic and Hazardous
Materials Agency (USATHAMA) Database
[After Tucker et al. 1985]

settling basin slurries, and unacceptable product from the manufacture process. Military and contractor personnel have compounded the pollution problem at some sites by discarding these TNT-containing waste materials for most of the past century (Major et al. 1992).

These past practices for the disposal of TNT and TNT-containing wastes have left behind a significant soil and sediment contamination problem. Frequently at contamination sites, the soil TNT concentration is measured in the thousands of parts per million (ppm) and therefore in sufficient quantity to be capable of causing significant and undesirable environmental effects. Additionally, the greater TNT contamination is usually stratified in the uppermost soil horizons making it readily accessible to a wide assortment of environmental organisms, including humans.

TNT presence in the environment represents an enormous hazard because of the high energy properties but, equally ominous is the biological toxicity of TNT and the environmentally produced TNT metabolites (Kaplan 1990). Further exacerbating the contamination problem, TNT in soils is long-lived; it has been detected in some soils 20 to 35 years after deposition (Roberts 1986, DuBois and Baytos 1991). This provides direct evidence that soils already

contaminated with TNT will supply a reservoir for continued environmental degradation far into the future.

Apprehensions about the eventual fate of TNT and TNT residues in soil are intensifying because of recent revegetation at some contaminated sites. It is feared that plant uptake of TNT could allow the introduction of TNT and hazardous TNT transformation products into the food chain (Harvey et al. 1990). Prior research shows this to be a valid concern because many plants uptake and convert TNT into many products. The severity of this is clear as TNT and some TNT products have been shown mutagenic by the Ames test (Kaplan and Kaplan 1982b) and because TNT is classified as an EPA Group C carcinogen (Gordon and Hartley 1989).

Besides worry of vegetative introduction of TNT into the food chain, the TNT must be eliminated from contaminated surface soils to prevent eventual pollutant migration into surface and ground waters. Pennington and Patrick (1990) theorize soil sorption alone will not effectively prevent TNT mobility through the lower soil layers and ultimately into underlying groundwater. Although TNT solubility at 20 °C is only about 128 mg/l, groundwater leachates resulting from munitions waste disposal have already been reported in several states (Spalding and Fulton 1988). While surface water contamination must remain a concern, the contamination of groundwater must be prevented; it is much more difficult

and expensive to remove pollutants from groundwater. Further, while TNT is ultimately limited by biological degradation and photolytic breakdown in surface waters, decomposition in groundwater will be limited because there are fewer biological opportunities for degradation and no photo-decomposition (Ryon 1987).

Smock et al. (1976) report that TNT has a 96 hour LC_{50} of 2.58 mg/l for the fathead minnow. Similarly, Nay et al. (1974) showed a 96 hour mean tolerance limit of 2.6 mg/l TNT for the bluegill fish, *Lepomis macrochirus*. Won et al. (1976) determined that selected marine organisms, tidepool copepods (*Tigriopus californicus*) and oyster larvae (*Crasostrea gigas*), had a LC_{50} of between 5 and 10 mg/l TNT.

Both biotic and abiotic processes can transform TNT into products that are potentially toxic. Ideally, the breakdown products are CO_2 , water, and mineral elements. However, some intermediate TNT transformation products may be produced that are as toxic as the parent TNT material (Bollag 1992). The distribution of these resulting products is determined, to a large extent, by the mode of breakdown, aerobic or anaerobic.

The aerobic breakdown of TNT often promotes the production of unstable intermediates and can lead to the formation of covalent bonds with other intermediates.

Linked intermediate products are more complex than the parent material and may be more hazardous. On the other hand, the anaerobic metabolism of TNT likely avoids some problems associated with the complex intermediate products and usually proceeds rapidly to the amino compounds. Unfortunately, these amino breakdown products are not totally safe. They are less toxic to some organisms than the original TNT, but are more toxic to others (Amerkhanova and Naumova 1979, Funk et al. 1993).

Many potential solutions have been researched over the years for the elimination of TNT from the environment. Most have proven costly and inefficient with few capable of handling the elevated TNT concentrations found at actual contamination sites. Also, many of the potential solutions investigated have generated undesirable intermediate and final products or proceeded at too slow a rate to be practical for the disposal of large quantities of TNT contaminated materials. Current TNT-soil contamination remediation technology and emerging research efforts center on: incineration, compost, surfactant, and biological systems.

Of the current technologies, incineration is the state-of-the-art remediation method for TNT contaminated soils. It is the only proven process for the complete elimination of TNT from contaminated soils. Public perception, however,

has become anti-incineration and most states have curtailed incineration operations. Additionally, incineration has proven costly, energy intensive, and ecologically destructive.

A compost process may eventually demonstrate the efficacy of being a viable treatment alternative for the remediation of TNT soil contamination. However, the compost degradation mechanism is not firmly established and further study is required. A full understanding of the intermediary metabolism of the contaminant is a very important part of the design of any effective remediation procedure (Crawford et al. 1992).

Another possible treatment method is the use of a surfactant to solubilize the contaminate materials into an insoluble complex. While this method could also prove to be an achievable treatment alternative, the process creates fundamental problems that must be overcome. These problems include: increased mutagenicity of the complexed materials, excessive amount of surfactant required, high pH leaching of soil organic components, and failure of the method to complex the amino derivatives (Kaplan and Kaplan 1982c).

Therefore, the most promising avenue for future technology centers on biological remediation. Crawford et al. (1992) declares that the biological remediation of contaminated soils is the more attractive alternative when

compared to physical remediation methods. Other researchers agree with this assessment based on the ability of biological remediation to provide a more earth-friendly manner of remediation and its potential for in situ treatment.

A variety of biological systems are known to have the ability to transform TNT into numerous products; some products are more complex than the parent material and some are equally toxic. The initial step in the metabolism of TNT by most biological systems involves the stepwise reduction of the nitro groups, through the nitroso and hydroxylamino, to the amino reduction products (McCormick et al. 1976). Under aerobic conditions, the biological mediated reduction of TNT usually stops at monoaminodinitrotoluene or diaminonitrotoluene. Apparently, the biological reduction of the third nitro group occurs only under anaerobic conditions (Roberts et al. 1992, Funk et al. 1993). Consequently, depending upon the reducing potential of the system, one, two, or three of the nitro groups may be biologically reduced to amino groups (McCormick et al. 1976).

Attempts to find a biological system capable of TNT elimination process has been stymied at this point for several years because the biological reduction of TNT to the amino compounds represents only a superficial modification

of the molecule and not true decomposition (McCormick et al. 1976). As the amino compounds are toxic and very water soluble, their generation as end products is highly undesirable because of the increased potential for product migration and groundwater contamination. Complete remediation of TNT contaminated soil requires the conversion of TNT to its amino derivatives and then the conversion of these derivatives to nontoxic products (Funk et al. 1993).

Previous research shows TNT to be resistant to biodegradation by most bacteria and fungi even at TNT concentrations significantly below those found at most contamination sites. However, with the large number of contaminated sites requiring some form of remediation, a significant effort has evolved to design and implement more ecologically-friendly and cost-effective means of soil remediation for many xenobiotic compounds, including TNT. The key criteria for any realistic remediation scheme are cost and time requirements and amount of ecological destruction. Obviously, any remediation method that can lessen these parameters will be advantageous. A rational technique incorporating these criteria for remediation of TNT contaminated soils, biological or otherwise, has yet to be developed and put into operation.

Using plants as the central means of biological remediation for TNT contaminated soil has been mostly

overlooked and not extensively researched. Most previous research efforts involving plants were aimed at learning how the contamination adversely affected the plant or what plant-produced metabolites became available for ingestion into the food chain. Nonetheless, initial EPA laboratory research using plant tissue extracts and pond sediments show a plant-produced nitroreductase enzyme can biologically reduce TNT to triaminonitrotoluene, the third and final amino reduction product (Ou 1994b). Further, select plants have a lactase enzyme that catalyzes the oxidation of the reduced amino products to give ring opened products and subsequently mineralization of TNT (Wolfe et al. 1994). In view of this, a phyto-remediation system for TNT could provide the nucleus for remediation.

Scope and Objectives

The purpose of this research was to develop and carry out a bench scale study of the biochemical remediation of a TNT contaminated soil using phyto-remediation. As such, the role of the selected vegetation, Parrotfeather, in the remediation of an actual TNT contaminated soil was investigated. Specifically, the breakdown of the recalcitrant nitro functional groups of TNT into more labile amino functional groups that allows other transformation

processes to cause further attenuation of the pollutant. Much of the initial laboratory and column studies using plant enzymes were conducted at the EPA Research Laboratory, Athens, Georgia (ERL-A). Additional laboratory studies were also performed at the ERL-A to quantify and identify specific reduction products resulting from this research.

This bench scale project could prove critical to the successful progression from laboratory investigation to initial full scale field study. Therefore, the experimental field design emphasizes scenarios that are reproducible in actual field situations or impacts potential field parameters.

The selected research field site was the Alabama Army Ammunition Plant (AAAP) located in Childersburg, Alabama. TNT contaminated soil was mined on-site and from a selected "hot spot", a localized small area of high TNT concentration. Additionally, a local beaver pond contained adequate quantities of the experimental plant selected for this research. Water, taken from the same beaver pond, provided the necessary environment for the plants. The relative location within the industrial area of the field experiment, soil contamination, and beaver pond sites are shown in Figure 1.2. Particular attention focused on obstacles encountered that might significantly influence a full scale field study.

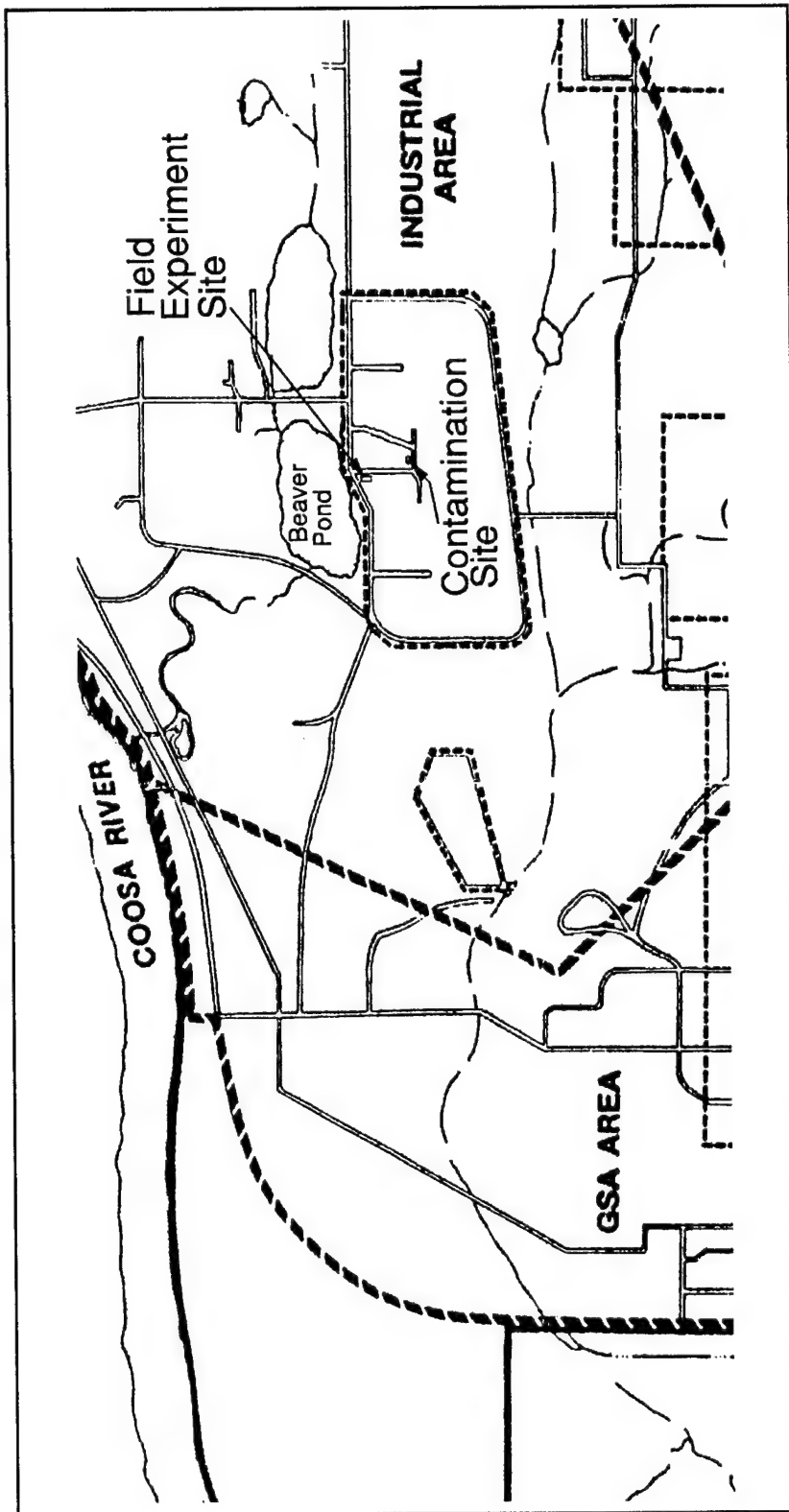


Figure 1.2

Field Experiment, Soil Contamination, and Beaver Pond Sites

Site Assessment Summary

A site assessment was conducted at the AAP, formerly the Alabama Ordnance Works, by the Army in 1978. This summary is extracted and condensed from that document. The AAP was established in 1941 on 13,233 acres of land located in Talladega County, four miles north of Childersburg and forty miles southeast of Birmingham, Alabama. A U.S. Army Corps of Engineers local vicinity map is shown as Figure 1.3. Explosives were manufactured at the AAP from 1943 to 1945; the type and quantity of the major compounds are listed in Table 1.1. Although the government began to surplus property as early as November 1945 the bulk of the property was placed on standby status from January 1946 to October 1975.

The AAP, designed to operate on the older batch mode of production was costly to maintain in standby status and declared excess to Army needs in 1973. However, about 2000 acres required some form of decontamination and could not be sold as it was consigned to the continued responsibility of the Army. Areas of possible TNT contamination included the red water settling basin and drainage ditch, the underground drainage lines, and surface contamination in the TNT areas.

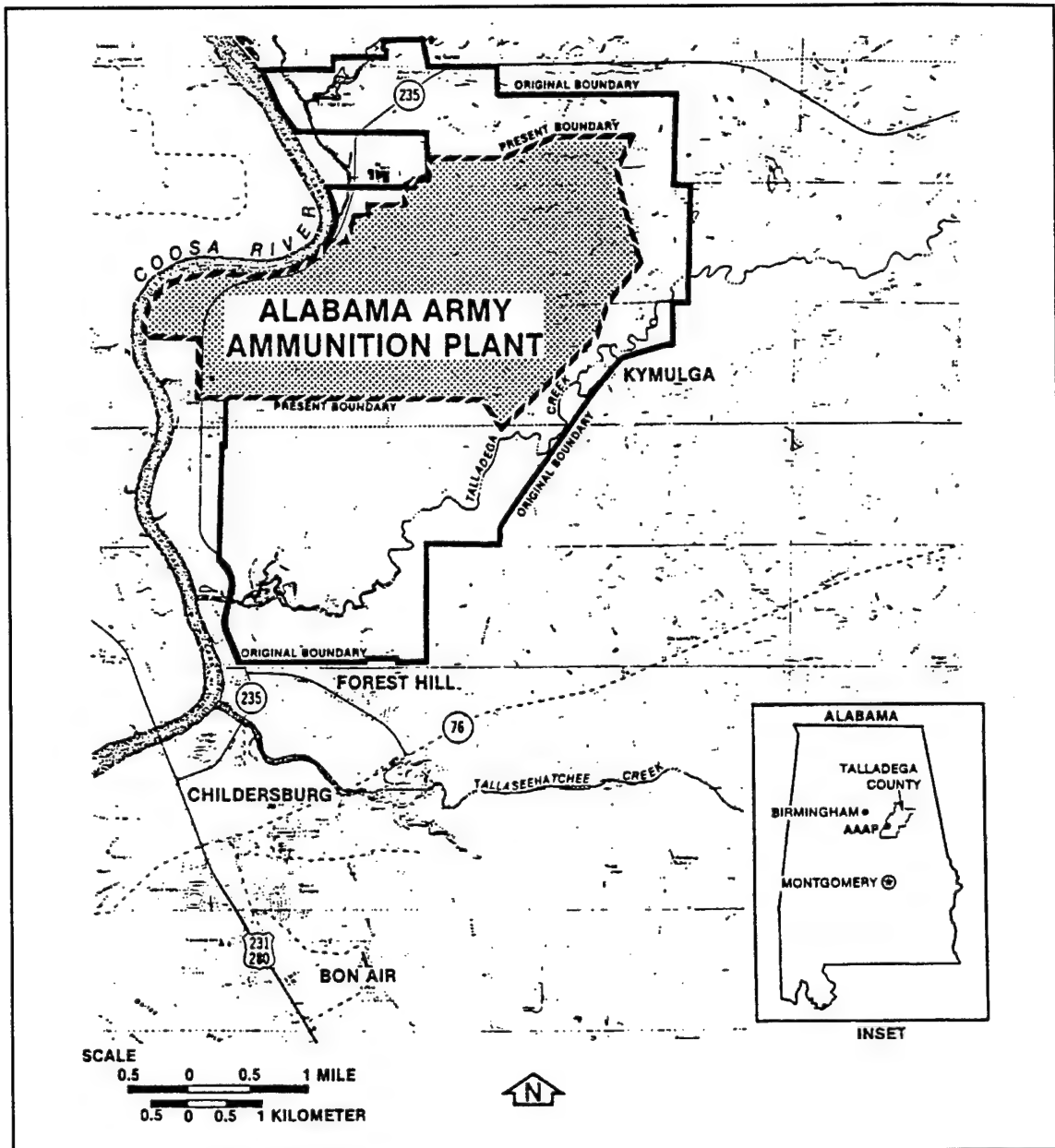


Figure 1.3
Vicinity Map of the AAAP

Table 1.1

Explosives Manufactured at AAAP from 1943 to 1945

Type Explosive	Quantity (million pounds)
Trinitrophenyl Methylnitramide (Tetryl)	57
Trinitrotoluene (TNT)	411
Dinitrotoluene (DNT)	39
Diphenlyamine (DPA)	10
Rifle Powder	127
Cannon Powder	375

Drainage

The main portion of surface runoff drains to the west or southwest toward the Coosa River. This is shown in the surface drainage sketch taken from the Army report and included as Figure 1.4. Also, the regional direction of groundwater gradient within the plant area is westward toward the Coosa River. Of the limited surface water quality data available during production operations, waste flow to the Coosa was controlled for pH and acidity using ground limestone (calcium carbonate). There were no records suggesting that other parameters were analyzed at the time of the 1978 report and no groundwater data was included in the report.

Soil

According to the Army report, the industrial plant area is underlain by dolomite and limestone of the Knox Group of Cambrian-Ordovician Age. Also, the average depth to the limestone bedrock surface varies from 40 to 60 feet with rock pinnacles projecting into the overburden; the subsurface bedrock is karstic in nature. The soil column from the surface down to the six-foot depth was listed as soft to hard, tan to reddish brown silty clay with organic components, sand, and containing a trace of gravel. Further, soils located within the production areas occur in

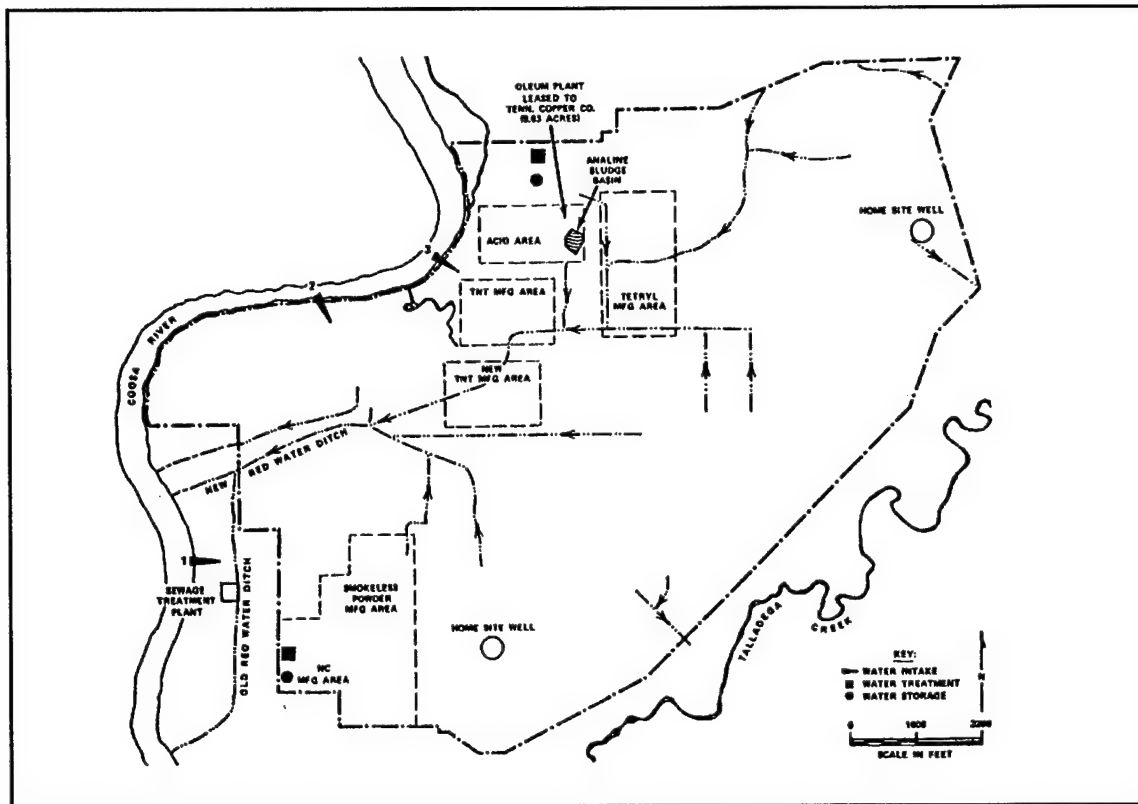


Figure 1.4

Surface Drainage and Site Overview

heterogeneous layers with low permeability to vertical migration of water. According to the 1978 assessment, the major contamination leaving the plant would be by surface routes and contamination leaching into the subsurface was of such a small quantity that the possible migration off post via groundwater was remote.

Contamination

Figure 1.5 is also taken from the Army report and graphically depicts the field test range and various areas of potential contamination within the site. As mentioned in the 1978 site assessment, there were visible traces of high explosives observed on the ground in both TNT production areas numbered six and seven. Also, smokeless powder pellets, used in rifle ammunition, were found on the surface in area two, reportedly due to spillage during packaging for transport. The burning grounds, areas numbered 12 and 16, were also suspected to be contaminated due to explosives disposal, however, the report did not mention any observable contamination. As other waste materials were also burned at this location, only burn residuals would be expected.

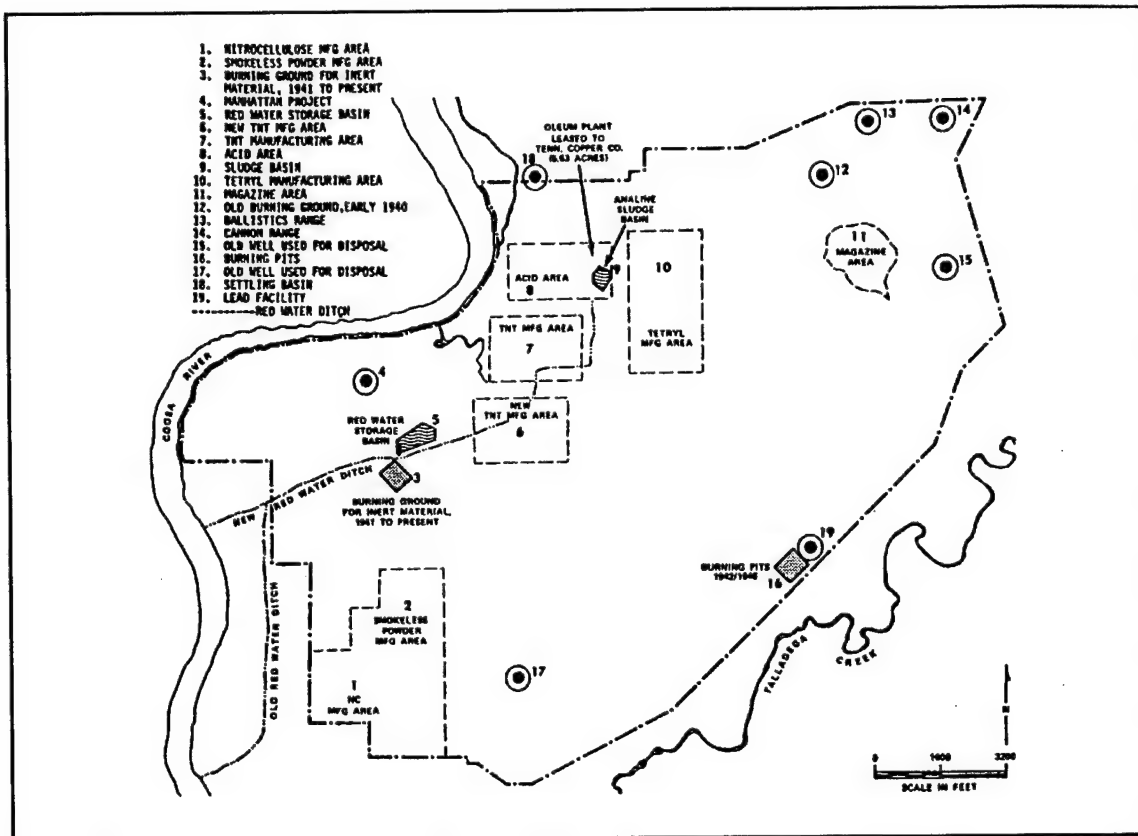


Figure 1.5

Field Test Range and Contamination Map

Methodology

This research performs a series of field experiments designed to detect and elucidate the problems associated with the selected phytoremediation scheme using the plant parrotfeather. Further clarification was provided by a series of experiments performed in the laboratory. These laboratory experiments were designed and performed to better quantify the field results and predict future avenues of investigation.

Organization

This dissertation is organized into six chapters. A brief overview of each chapter follows: Chapter One outlines the contamination problem, previous research avenues, and the purpose and methodology of this research; Chapter Two covers, in more depth, the background and information generated by previous research in TNT contamination and remediation efforts; Chapter Three details the methods and materials used in both field and laboratory experiments; Chapter Four details the field experiments; Chapter Five details the laboratory experiments; and Chapter Six provides a summary of the research and includes suggested additional research avenues.

CHAPTER 2

TRINITROTOLUENE (TNT) LITERATURE REVIEW

The literature concerning TNT, its environmental contamination, and a myriad of potential degradation opportunities are extensive and cannot be exhaustively reviewed here. As such, the purpose of this review is to synopsise the data germane to this specific research effort.

TNT transformation pathways are emphasized where possible to delineate the intermediate product formation. Thus, this review encapsulates the soil TNT contamination problem; how it came about, why it is hazardous, and what studies have been undertaken to eradicate it from the environment.

General Information

Conventional Military Explosive

TNT is the predominant conventional energetic material used for most of this century by all branches of the United States military. It is the high explosive adopted by the Army for the entire Department of Defense in 1904 and used in a wide variety of munitions (Nay et al. 1974). TNT's

longtime popularity results from its material attributes of low melting point; stability; low sensitivity to impact and friction; and the relatively safe methods of manufacture (Roberts 1986, Nay et al. 1974).

TNT Properties

TNT occurs in six isomeric forms: 2,4,6- or α ; 2,3,4- or R; 2,4,5- or γ ; 3,4,5- or δ ; 2,3,5- or ϵ ; and 2,3,6- or η . For the most part, the α or 2,4,6- isomer is the one of primary military interest and is normally designated simply as TNT. The other five, "meta," isomers are formed as by-products during TNT production (Roberts 1986) and are removed during the manufacture purification process.

At 20 °C TNT exists as colorless to yellow monoclinic needles or orthorhombic crystals (Carper et al. 1982, Connick et al. 1969). Elemental analysis of TNT yields 37.01% C, 2.22% H, 18.50% N, and 42.27% O (Roberts 1986). Selected TNT properties are listed in Table 2.1.

TNT Manufacture

TNT is manufactured by the nitration of toluene in a step-wise, three-stage process proceeding first to the

Table 2.1
Selected TNT Properties
[After Roberts 1986]

Molecular Formula	$C_7H_5N_3O_6$
Molecular Weight	227.13
Melting Point	80.1 °C
Density	1.654 g/cm ³
Vapor Pressure	0.046 mm Hg at 82 °C
Viscosity	8 cP at 99 °C
Heat of Formation	0.293 KJ/g
Heat of Combustion	15.02 KJ/g
Specific Heat	1.38 J/g °K
Thermal Conductivity	0.54 W/m °K
* Water Solubility (20°C)	100 mg/l

* Note: Different literature lists the TNT solubility in water at 20° C to vary from 100 to 140 mg/l. A more likely value of 128 mg/l was used in this study.

mono-nitrotoluene, then to the di-nitrotoluene, and finally to the tri-nitrotoluene compound (Roberts 1986, Nay et al. 1974). This reaction sequence for the formation of 2,4,6-Trinitrotoluene is outlined in Figure 2.1. A crude basic TNT results from the three-step nitration process and is then purified by contacting it with a sodium-sulfite (sellite) solution to remove the unsymmetrical TNT "meta" isomers. This same purification operation is also needed to remove various oxidation products that result during the nitration process (Nay et al. 1974).

TNT was manufactured exclusively by a batch process at the army ammunition plants (AAP) until 1968 when the countercurrent continuous flow technology replaced the less efficient batch method (Roberts 1986, Nay et al. 1974). Greater efficiency resulted because the new technology more completely utilized the chemical potential of the raw materials (Nay et al. 1974). Also, because many of the TNT complexed materials could be removed from the waste stream making the resulting condensate wastewater more susceptible to biological degradation (Nay et al. 1974). Still, the manufacturing waste effluent contained significant concentrations of TNT (Traxler 1974). In addition to manufacturing waste streams, effluent from the load, assemble, and pack (LAP) operations also contained TNT concentrations approaching saturation. Both AAP and LAP

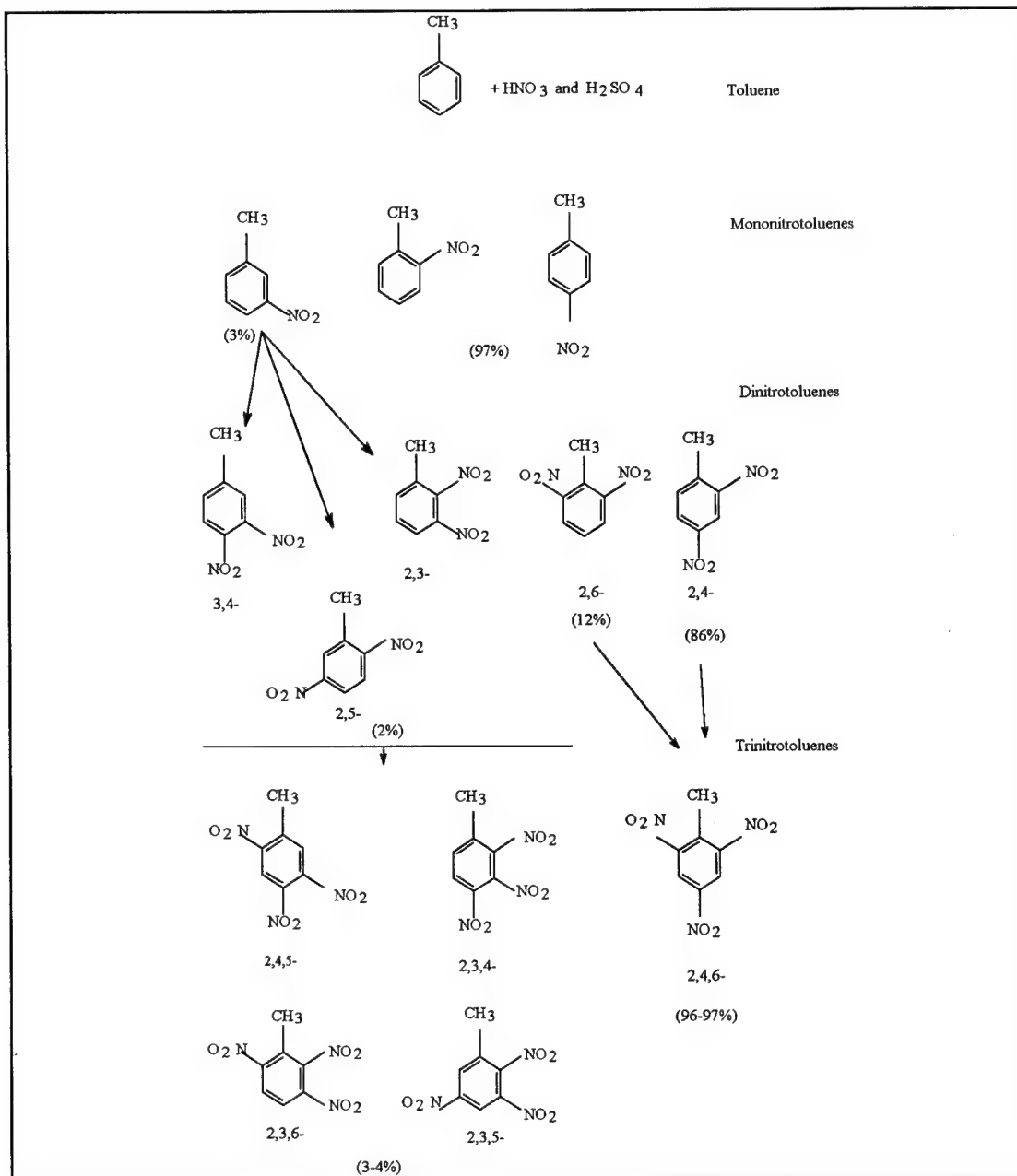


Figure 2.1

Reaction Sequence for the Formation of 2,4,6-Trinitrotoluene

[After Schott and Worthley 1974]

operations resulted in the introduction of significant amounts of TNT into the environment via waste effluent discharges. This has caused soil, water, and sediment TNT contamination at many military sites (Roberts 1986, Fernando et al. 1990, Spiker et al. 1992). Extensive research shows this TNT contamination to be long lived and environmentally hazardous; TNT is toxic, mutagenic, and carcinogenic.

Toxicity

TNT contamination represents an environmental hazard because of the high energy properties of the material but also due to its toxicological effects on a number of biological systems (Kaplan 1990). Equally toxic are the by-products resulting from the TNT manufacturing process, such as 2,4-Dinitrotoluene (DNT) and the transformation products created by the degradation of TNT in the natural environment. The TNT transformation products are, in many instances, equally or more hazardous than the parent TNT (Newell et al. 1976, Harvey et al. 1990). A list of the common TNT transformation products with abbreviated name and a mechanism responsible for the product formation is presented as Table 2.2.

The 2,4DNT is included in this table because it is the predominant by-product resulting from the manufacture of TNT (Spanggord et al. 1991). It is listed as a priority

Table 2.2
Common TNT Transformation Products, Abbreviations, and a Mode of Generation

TNT Transformation Product	Abbreviation	Generation Mode
2-amino-4,6-dinitrotoluene	2ADNT or 2A	Biological Reduction
4-amino-2,6-dinitrotoluene	4ADNT or 4A	Biological Reduction
2,4-diamino-6-nitrotoluene	2,4DANT	Biological Reduction
2,6-diamino-4-nitrotoluene	2,6DANT	Biological Reduction
4-hydroxylamino-2,6-dinitrotoluene	4HA	Biological Reduction
2,4-dinitrotoluene	2,4DNT	Production by-product
2,6-dinitrotoluene	2,6DNT	Production by-product
2-hydroxylamino-4,6-dinitrotoluene	2HA	Biological Reduction
2,2',6,6'-tetranitro-4,4'-azoxytoluene	4,4'Az	Biological Reduction
4,4',6,6'-tetranitro-2,2'-azoxytoluene	2,2'Az	Biological Reduction
2',4',6',6'-tetranitro-2,4'-azoxytoluene	2,4'Az	Biological Reduction
2,4,6-Triaminotoluene	TAT	Biological Reduction
1,3-Dinitrobenzene	DNB	Photolytic Breakdown
1,3,5-Trinitrobenzene	TNB	Photolytic Breakdown
2,4,6-Trinitrobenzaldehyde	TNBAL	Photolytic Breakdown

pollutant by the United States Environmental Protection Agency (EPA) due to its toxicity (Keith and Telliard 1979). Also, its environmental breakdown is analogous to TNT and yields similar reduction compounds.

Environmental breakdown of TNT and DNT by simple reduction of the nitro groups to corresponding amino groups on the ring does not eliminate the toxicity of the TNT molecule (Funk et al. 1993). These amino derivatives are less toxic to some organisms, but are more toxic to others (Amerkhanova and Naumova 1979).

However, the overall amino product toxicity may not be as great as the parent TNT. Hankenson and Schaeffer (1991) found TNT 20 to 50 times more toxic than diaminonitrotoluene (2,6DANT) which was used for the comparison because it is one of the primary reduction products created by the biological metabolism of both TNT and DNT. The particular microtox test used for their research was a bioassay to measure the reduction in chemiluminescence by the marine bacterium *Photobacterium phosphoreum* when it was exposed to the toxic materials (Hankenson and Schaeffer 1991).

Human

TNT is toxic to humans, extent of toxicity is a function of exposure route. It is readily absorbed when

orally, when contacted with the skin, or when inhaled into the respiratory passages as dust. Skin contact is the primary route of entrance into the body, with oily, sweaty skin greatly facilitating absorption. Thus, the palms provide the site where most absorption occurs (Hamilton 1921, Zakhari et al. 1978, El-hawari et al. 1981, Roberts 1986).

Most explosives, usually organic nitro compounds, can cause pathologic symptoms in man (Osmon and Klausmeier 1973). Thousands of people, primarily in the TNT processing occupations, have been exposed to TNT in various forms and concentrations. Some of these have been long term exposures occurring over periods of many years. Occupational exposure evaluation of these individuals has provided the evidence that TNT adversely affects numerous human body organs (Jaffe et al. 1973, Zakhari et al. 1978). In addition to long term exposure problems, test subjects involved in short term TNT research often developed a rash and even limited TNT exposure usually evidences some decrease in hemoglobin and red blood cell counts (Zakhari et al. 1978).

Human exposure to TNT is known to cause pancytopenia. This is a disorder of the blood-forming tissues characterized by a pronounced decrease in the number of leukocytes, erythrocytes, and reticulocytes in humans and other mammals (Fernando et al. 1990). TNT has been shown to

cause liver damage as well as anemia in humans (Sax 1957, Won et al. 1976). Liver damage likely results because it is the primary organ responsible for detoxification of TNT in mammals (Ellis et al. 1980).

There were 4,000 documented cases of TNT toxicity resulting in 580 deaths from occupational exposure during World War I (Pennington 1988, Roberts 1986). Urbanski (1964) has summarized the recorded cases of TNT poisoning of munitions workers through the early 1960's. He found that at one munitions facility alone, 17,000 cases of TNT poisoning were recorded of which 475 were fatal.

Fortunately, the introduction and use of industrial hygiene measures reduced the hazard during World War II to only 22 fatalities. These deaths were attributed to overexposure of workers to TNT dust by inhalation (McConnell and Flinn 1946).

Death from TNT exposure most frequently results from toxic hepatitis, aplastic anemia, or spleen, kidney, or other major organ failure. Also, death may result from toxic effects to the hematological and nervous systems (El-hawari et al. 1981, Jaffe et al. 1973). The major causes of mammalian death resulting from TNT exposure are hepatotoxicity, leading to yellow atrophy of the liver, and aplastic anemia associated with bone marrow hypoplasia (El-hawari et al. 1981, Jaffe et al. 1973, Urbanski 1964).

There is evidence of extensive TNT metabolism prior to elimination in urine but no evidence of unchanged TNT in the urine of humans. Significantly, the products found in urine of people that have been exposed to TNT are predominantly the same compounds as those identified in the urine of animals exposed to TNT (Channon et al. 1944, Lemberg and Callaghan 1944).

Animals

Studies with rats, mice, rabbits, and dogs show that TNT administered by mouth, adsorbed through the skin, or lung adsorbed from inhaled dust is readily distributed and extensively metabolized by the animals (El-hawari et al. 1981). It is usually excreted in the urine as metabolites with only minute amounts of the parent TNT present. Further, it is dispersed throughout the body of the animal to include the blood, liver, kidneys, lungs, spleen, brain, fat, and muscle (Hodgson et al. 1977, El-hawari et al. 1981, Ellis et al. 1980). No single point of accumulation exists and only trace amounts are found in most tissues (El-hawari et al. 1981, Ellis et al. 1980). Research analyses show that the storage and retention of TNT differs by species and route of administration (El-hawari et al. 1981).

Cerebellar lesions, hemolytic anemia, hypercholesterolemia, hepatomegaly, splenomegaly, and testicular atrophy were the major toxic effects resulting from an oral TNT diet conducted on Fisher 344 rats for 13 weeks (Levine et al. 1981). Thus, the liver, testes, and blood were the body components most greatly affected (Levine et al. 1984). On a longer term, 24 month study, the major toxic effects were anemia with secondary splenic lesions, hepatotoxicity, and urogenital lesions. Also observed were lesions of the liver, kidneys, and urinary bladder (Furedi et al. 1984).

Other Organisms

Exposure to TNT and photo-irradiated wastewater containing colored complex compounds of TNT (TNT_{cc}) have been shown to be toxic or inhibitory to a large variety of biota. This includes certain green and blue-green algae such as, *Selenastrum capricornutum*, *Microcystis aeruginosa*, and *Chlamydomonas reinhardi*. Also significantly affected are tidepool copepods like *Tigriopus californicus* and oyster larvae identified as *Crassostrea gigas* (Won et al. 1976).

Even low TNT contaminate levels can produce adverse effects on many organisms. This is evidenced by the algal research where the *S. capricornutum* algae was growth

inhibited at levels of only 5 mg/l with a no effect threshold of 3 mg/l and 9 mg/l for TNT and TNT_{cc}, respectively (Smith 1973, Smock et al. 1976). Although *M. aeruginosa* was growth inhibited at the higher level of 15 mg/l it showed a lower no effect threshold of only 1 mg/l for both TNT and TNT_{cc} (Smith 1973, Smock et al. 1976). Somewhat similar, the *C. reinhardi* showed little response to TNT concentration up to 1 mg/l but growth was completely inhibited at higher levels (Hudock and Gring 1970).

Fish are also very sensitive to TNT. Research results show TNT toxic to fathead minnows, *Pimephales promelas*, and bluegill fish, *Epomis macrochirus*, at concentrations of only 2-3 mg/l (Liu et al. 1976, Nay et al. 1974). Additionally, Osmon and Klausmeier (1973) suggest a mean tolerance limit of 2 to 3 mg/l for several other freshwater fish. The *P. promelas* has displayed a higher sensitivity to the photochemical irradiated transformation products of TNT found in pink water than to the pure TNT in solution (Smock et al. 1976). Research by Smith (1973) determined the *P. promelas* 48 hour exposure limit to be 1.48 mg/l for the TNT colored complexes and 4.22 mg/l for the TNT.

Mutagen

TNT is mutagenic by the Ames test (Won et al. 1976). Additionally, there is evidence that TNT is a frameshift mutagen as determined by mutagenic assays carried out on a set of histidine-requiring strains of *Salmonella typhimurium*. Surprisingly, the TNT intermediate products were found nontoxic and nonmutagenic in one series of experiments (Won et al. 1976). Yet, subsequent research has determined condensate wastewater mixtures, composed of many TNT products, are mutagenic and photolysis actually increases the potency (Dilley et al. 1979).

Carcinogen

Exposure to TNT may pose a human carcinogenic risk. Dilley et al. (1979) state that TNT should be considered a potential carcinogen like many other aromatic nitro compounds. Research conducted on Fischer 344 rats showed urinary bladder papilloma and carcinoma (Furedi et al. 1984). Based on these and other animal studies, TNT has been classified as an EPA Group C carcinogen (Gordon and Hartley 1989).

Drinking Water Health Advisory

Health advisories (HA), while not regulatory standards, do state the specific concentration of a drinking water contaminant at which adverse health effects would not be anticipated to occur over some specific exposure duration. The HA for one-day and ten-day TNT exposure is 0.02 mg/l; it is 0.002 mg/l for a lifetime exposure level (Gordon and Hartley 1989).

TNT Contamination

Environment contamination by energetic organo-nitro compounds arising from ordnance manufacture and processing is an international problem. Significant TNT contamination problems currently exist in Europe, Southeast Asia, and Russia as well as the United States (Steuckart et al. 1994, Zappi 1994). Contamination has primarily resulted from: the land application of waste TNT materials, disposal by open burning and open detonation (Kaplan 1990, Major et al. 1992), groundwater seepage from settling ponds, and direct wastewater effluent discharge into the aquatic environment (Ryon et al. 1984). TNT contaminated and potentially contaminated Army sites within the boundaries of the continental United States are shown in Figure 1.1. Military

and contractor personnel have used some of these sites for most of the past century (Major et al. 1992).

The disposal of TNT-bearing materials, leading to the current environmental degradation at these sites, was according to the technology of the time and before the environmental impact was fully appreciated (Harvey et al. 1991). Both manufacture and LAP operations have provided ample opportunities for TNT and TNT products to contaminate the environment: air, water, and soil (Roberts 1986).

Air

Little information has been collected to quantify the airborne contamination and transport of TNT from munitions fabrication. TNT presence in atmospheric emissions was detected at two AAPs but not quantifiably measured (Carpenter et al. 1978). Apparently, this does not seem an area of great concern at this time. Also, the volatilization of TNT into the atmosphere from surface waters was considered environmentally insignificant (Spanggord et al. 1978).

Surface Waters

A series of aquatic field surveys of streams receiving munitions wastes from AAP operations have been conducted. Results show a certain loss of biological communities

downstream from effluent releases (Cairns and Dickson 1973, Fox et al. 1975, Griffiths et al. 1979, Jerger et al. 1976, Putman et al. 1979, Sanocki et al. 1976, Stilwell 1976, Sullivan et al. 1977, Weitzel et al. 1975). Although the identified problem levels were confined to times of high TNT production, the observed negative biological impacts could not be uncategorically assigned to TNT because there were other munitions products present in the effluent (Pennington 1988).

Research utilizing natural waters demonstrates that some natural water attributes mitigate the aquatic organism toxicity to TNT. Ryon (1987) found that increasing the pH, temperature, or sunlight exposure will decrease resulting TNT toxicity to aquatic organisms. Water hardness was also checked but was not found to affect the TNT aquatic organism toxicity (Ryon 1987).

The main avenue of TNT entrance into surface waters has been through wastewater effluent. Not all TNT related operations incorporated a settling process in the waste stream effluent; some wastewaters were discharged directly into local rivers and streams (Walsh et al. 1973). Although some solid materials may have been settled out before release, significant amounts of solid and dissolved TNT and related products were introduced directly into the aquatic environment from these operations.

Wastewater

Besides containing TNT, wastewater discharged from TNT manufacture and ordnance fabrication operations were often colored pink or red. Different chemical constituents, inherent to the specific type of wastewater, caused these characteristic colors.

Pink Water

One wastewater type, associated with the ordnance fabrication and assembly operations, consisted of wash waters containing TNT concentrations near the saturation limit (Kaplan et al. 1975). This type wastewater was generated from rinse water used to remove residual explosive residues from equipment, rejected shells, and the interior surfaces of facilities (Harvey et al. 1990, Ruchhoft et al. 1945). A single shell loading plant generated roughly 50,000 gallons of this type of wastewater per day (Ruchhoft et al. 1945). Although the actual TNT concentration in solution was only about 100 mg/l, the sheer volume of wastewater insured a sizable contribution to the pollution problem (Walsh et al. 1973).

These wastewaters were normally discharged from the ordnance facilities into channels, basins, and other surface structures. Initially a clear color, the water quickly underwent photochemical changes as it was exposed to

sunlight (Smock et al. 1976). The aqueous TNT incorporated in the water was photochemically transformed into a labyrinth of compounds and isomers commonly called "pink water." The nature and exact composition of pink water have not been determined (Smock et al. 1976).

Sometimes, the final water washing following the sellite purification step in TNT manufacture was also called pink water (Walsh et al. 1973). Yet, the distinctive pink coloration characteristic of the wastewater TNT photolytic decomposition was primarily associated with the LAP operations (Roberts 1986).

Red Water

Instead of receiving its coloration from photolytic breakdown of TNT, the "red water" color was formed during the purification process of TNT manufacture (Roberts 1986). It contained sulfonates derived from TNT isomers and other more complex chemicals in various concentrations (Rosenblatt et al. 1971, Nay et al. 1974).

Also, the red water TNT-related components differed by method of manufacture: batch or countercurrent continuous flow (Nay et al. 1974). In either case and regardless of the manufacture process, the wastewater consisted of a great number of compounds. Spanggord et al. (1982) identified over 30 specific nitroaromatic compounds in the wastewater

from one countercurrent continuous flow process. A single TNT manufacturing facility generated as much as 500,000 gallons of this wastewater each day (Traxler 1974).

A process for the destruction of TNT red water was initiated at the Radford Army Ammunition Plant in the 1980's (Higgins 1983). As a sulfite recovery operation, the process used a red water furnace to incinerate and then chemically convert the waste to a sodium sulfite solution. This solution was then recycled for reuse in the TNT purification process (Higgins 1983).

Still, another type of wastewater resulted from this recovery operation through the distillation of red water. Called condensate wastewater, it was composed mainly of nitroaromatic by-products of TNT, 30 of which were identified (Liu et al. 1983). Liu et al. (1983) reported this wastewater had overall a low acute toxicity that decreased with photo irradiation. However, they did identify three organic components of the wastewater to be acutely toxic: 2,3,6TNT, 2A3,6DNT, and 1,3,5TNB.

Wastewater Handling

Schott et al. (1943) analyzed the combined liquid waste from manufacturing plants in an early research effort. These composite waste streams were made up of acid wash waters, red water, and cooling waters combined. Their

findings showed the wastewaters were characterized by low pH, high color, high total solids, high sulfates, and high oxygen consumed values. Later research on the continuous flow wastewaters showed similar results (Nay et al. 1974). Also, Nay et al. (1974) observed that the pH neutralization, required to make the manufacture wastewaters more amenable to biological treatment, increased the wastewater color values from about 200 standard color units to around 7000. They also found the wastewater more resistant to treatment, by both biological means and physical adsorption processes, as the color values increased (Nay et al. 1974).

Many research investigations were conducted to devise a better way of handling wastewater effluent. By the late 1970's, carbon adsorption was showing success in the treatment of pink waters (Roth and Murphy 1978). Also, Andrews and Osmon (1975) researched the use of ultraviolet light (UV) as a treatment method to make TNT-containing waters more amenable for subsequent biodegradation. Then, Andrews (1980) showed that, on a pilot scale, UV together with a hydrogen peroxide oxidant achieved a high degree of TNT removal without production of troublesome by-products. Also, Liu et al. (1976) found that UV exposure reduced the toxicity of LAP, condensate wastewater, 2,4DNT and TNT. Red water toxicity, on the other hand, was not affected by irradiation (Liu et al. 1976) and by the mid 1980's much of

the red water was being incinerated in sulfite recovery operations.

Unfortunately, not all munitions plants were capable of incinerating the large volume of finishing plant wastewaters so the excess volumes were sometimes discharged directly into nearby rivers and streams (Walsh et al. 1973). The standard practice for handling most TNT-saturated production wastewaters was to channel the effluent into drainage ditches to convey the flow into holding lagoons or evaporation-percolation ponds (Roberts 1986, Harvey et al. 1990). However, these on-site wastewater settling structures were normally constructed to discharge overflows directly into local streams (Ruchhoft et al. 1945). Because of this, wastewater discharge from AAPs was the primary way that TNT entered the aquatic environment with concentrations ranging from 0.05 to 178 mg/l (Ryon et al. 1984). Exacerbating the contamination problem, TNT demonstrates an inhibitory effect on the biochemical processes of self-purification in water at concentrations greater than 1 mg/l (Rogovskaya 1951).

Groundwater Contamination

Besides surface water pollution, leachates from the disposal of munitions wastes also pose a huge problem and have been reported in several states (Spalding and Fulton

1988). TNT persistence in surface waters is ultimately limited by biological degradation and photolytic breakdown. However, as a solid material or dissolved in groundwater, TNT will not decompose for a long time (Ryon 1987).

Frequently, nitrate and nitrated organics are found in groundwater near ordnance facilities. These organics can often be traced back to a TNT manufacturing site or shell loading operation (Spalding and Fulton 1988). Pereira et al. (1979) conducted a characterization study of groundwater contamination at a depot in Nevada. They found that shallow ground water, under and down gradient, was contaminated with TNT and other nitrogen bearing compounds as a result of percolating wastes from old disposal beds.

Soil Contamination

As operations ceased at many production sites, explosive residues and their transformation products have accumulated in large areas of soil formerly occupied by settling structures (Harvey et al. 1990, Harvey et al. 1991). This has left highly TNT contaminated soil at many of these locations.

In addition to heavy soil contamination from evaporation of settling ponds, TNT and TNT-containing solid materials have been land deposited over the years. These deposits include: the land application of the waste solids

resulted from an unacceptable batch in the manufacture process, the site of deposit for sludge and spoils dredged from settling lagoons and drainage ditches, the entombment of many such structures upon closure of operations, and excess or contaminated stores burned or dumped as waste. Because these wastes were usually discarded at the surface level and as TNT is not very water soluble, the higher levels of TNT contamination remain in the uppermost stratum of the soil. Contaminated soil TNT concentrations are usually measured in the thousands of parts per million (ppm).

At sites of solids disposal, most TNT concentrations in the topmost soil layers result from the presence of concentrated crystalline TNT (Pennington and Patrick 1990). Cataldo et al. (1989) have learned that the half-life for soil TNT residues is from 4 to 10 years. They based these findings on CO₂ evolution rates for Burbank, Palouse and Cinebar soils. Combined with the fact that TNT has been detected in some soils 20 to 35 years after deposition (Sanocki et al. 1976, DuBois and Baytos 1991), it is easy to conclude that today's contaminated soil will continue to act as a repository for environmental contamination far into the future.

DNT Contamination

Another significant contamination product resulting from TNT production is 2,4-dinitrotoluene (DNT). It is the major impurity resulting from the manufacture of TNT and is used industrially in producing polyurethane foam (Spanggord et al. 1991). This material and other nonpolar nitroaromatic compounds are more resistant to microbial attack. Yet, Liu et al. (1984) have demonstrated a DNT nitro reduction similar to TNT as the anaerobic biotransformation of DNT into 2ADNT and 4ADNT proceeds through the nitroso and hydroxylamino compounds. Also similarly, they show that the nitroso compounds rapidly disappeared while the amino intermediates proved to be more stable (Liu et al. 1984). As the DNT was transformed into amino products without ring cleavage the resulting products are themselves potentially hazardous.

Spanggord et al. (1991) report the isolation of a *Pseudomonas* strain that can use DNT as the sole source of carbon under aerobic conditions. Apparently, the initial attack is via dioxygenase at the 4,5 position with the concomitant release of nitrite. Subsequent reactions complete the biodegradation with removal of the second nitro group as nitrite and subsequent mineralization. They theorize that this isolate may be useful in a treatment

system for nitrotoluene removal from contaminated soils. A treatment system has yet, however, to be developed.

Soil Contamination Migration

Short term accumulation and mobility of a xenobiotic contaminant in the natural environment is controlled by the chemical partitioning. Yet, with TNT, it is the biological and chemical stability of the parent and transformed products that has the greatest influence on persistence (Cataldo et al. 1989). TNT is known to be very persistent in the terrestrial environment.

Temperature Effects

The primary effects of temperature on TNT are twofold: first, the effect on the TNT solubility and secondly the effect on various microorganisms that might biologically transform the TNT. In the range between 15-30 °C (average agricultural temperatures) warmer temperatures increase both TNT solubility and microbial transformation rates.

Soil Interactions

Several TNT adsorption experiments have been conducted on soils with a wide range of parameters. TNT was found moderately mobile and its adsorption coefficient correlated

most with organic carbon content and cation exchange capacity in the soils tested by Roberts (1986). Similarly, Pennington (1987) found adsorption to be highly correlated with organic carbon content and cation exchange capacity, but also with the extractable iron and clay contents (Pennington 1987). Soils high in these properties may contribute to TNT environmental persistence by limiting the bioavailability of TNT and its products (Folsom et al. 1988).

Pennington and Patrick (1990) found that the adsorption and desorption phenomena occur to the same extent; TNT exhibits only limited soil adsorption. This means that soil sorption will not actively prevent the movement of TNT, in the solution phase, through surface soils and into the lower soil horizons (Pennington 1987, Pennington and Patrick 1990). Consequently, potential migration of TNT from these soils into groundwater and surface waters is a plausible concern (Pennington and Patrick 1990).

Apprehensions about the environmental fate of soil TNT residues are now intensified because of recent revegetation at some of the contaminated sites. Vegetative uptake could provide an avenue for the possible introduction of TNT, TNT microbial generated transformation products, and plant-produced TNT metabolites into the food chain (Harvey et al. 1990, Harvey et al. 1991). Counter to these concerns,

however, are the results from a study of game animals captured at the Alabama Army Ammunition Plant. No TNT or TNT related compounds were detected in the tissues of sampled game animals although the site has TNT contamination with vegetative encroachment into the contaminated areas. Game animals included in the study were: deer, rabbit, and quail (Shugart et al. 1991).

However, studies on TNT amended soils with plants grown to maturity shows TNT derived metabolite accumulation occurs in all tissues of the plant. Plant roots became the location of the greater TNT concentrations with the relative order of TNT accumulation being root >> stem > leaves >> seed and pod (Cataldo et al. 1989). Roots and stems are unlikely to be a prime food source for the game animals studied by Shugart et al. (1991) which might explain why no TNT related products were found in the animal tissues.

Total plant uptake of TNT was found to be inversely proportional to soil organic matter content (Cataldo et al. 1989). This could be due to attachment reactions in the soil medium as Kaplan and Kaplan (1983) present evidence of binding reactions between munitions waste compounds and soil organic matter. They suspect that the active binding species are the DANT biological reduction products.

Soil Redox

Although sorption of nitroaromatics to soil organic components does occur, sorption to inorganic components predominates (Leggett 1991). Also, experiments using sterile soil systems show that the sorption process is abiotic in origin (Cataldo et al. 1989). Nitroaromatics readily participate in donor-acceptor interactions; they can both donate and accept electrons depending on the environment (Leggett 1991). TNT in soil-water systems may become an ultimate recipient of charge thereby causing its reductive transformation and subsequent covalent bonding to soil organic matter components (Leggett 1991).

The redox potential in aqueous systems affects the oxidation states of many elements and is limited only by the oxidation-reduction limits imposed by the stability of water. The limit of oxidizing conditions in aqueous systems is the oxidation of water to molecular oxygen; the limit of reducing conditions is the reduction of the hydrogen ion to molecular hydrogen (Bohn 1971). Yet, natural systems rarely reach oxidation-reduction equilibrium because of the continual addition of electron donors via oxidizable organic compounds (Bohn 1971) and kinetic limitations.

Redox conditions in living systems are nonhomogeneous and are usually measured as mixed potentials (Bohn 1971). Further, they are pH dependent and measurements are

qualitative, thus, only meaningful when subject to interpretation by a knowledgeable observer (Bohn 1971). Research results by knowledgeable investigators show that the redox potential and pH exert no effect on adsorption, desorption, or transformation of TNT in soil samples collected from 13 contaminated sites (Pennington 1988, Pennington and Patrick 1990).

TNT Transformations in Soil

Kaplan et al. (1985) researched the effects of a variety of environmental factors on the TNT fate in soils using three soil-TNT concentrations by weight: 0.1%, 1%, and 10%. They found the initial TNT concentration to be the most significant factor affecting the rate of transformation. This is likely due to variations in levels of microbial activity associated with the variations in TNT concentration; the greater the initial TNT concentration the lesser the microbial activity. The next most significant factor associated with the TNT transformation in soil was the absence or presence of soil organisms and the incubation temperature. They also found the moisture level to be a less important environmental factor with the amounts of organic matter and oxygen level the least significant of the factors studied.

These results explain why the TNT found in soils is often tenacious and difficult to eliminate. A greater TNT concentration, fewer active organisms, colder temperature, and dryer soil, all result in TNT persistence in an unaltered state (Kaplan et al. 1985).

TNT is one of the most frequently found contaminants in soils collected from ammunition plants, depots and arsenals (Walsh and Jenkins 1992). When TNT is present in a soil, TNB, DNT, and ADNT isomers are also generally present at detectable levels (Walsh and Jenkins 1992). While the DNT is a manufacturing by-product, the others are transformation products; TNB results from photodecomposition and ADNT is attributable to biological metabolism (Walsh and Jenkins 1992).

TNT Elimination

The fate of TNT in soil and/or water is ultimately controlled by a variety of factors. It is significantly influenced by biological reductions and sunlight exposure.

Biological Reduction

Many investigations have examined the metabolism of TNT in bacterial, animal, and plant systems. Metabolic degradation of TNT in these systems, for the most part,

embodies a stepwise reduction of the nitro groups. The reaction proceeds from the nitro group, through the intermediate nitroso and hydroxylamino, to the amino group (McCormick et al. 1976). Yamashina et al. (1954) relate that each nitro group reduced to the corresponding amino group requires three moles of H_2 (Kaplan 1990, McCormick et al. 1976). For the most part, this nitro group reduction is nonspecific and performed by many bacterial species (Funk et al. 1993). Figure 2.2 is an illustration of this universal reduction pathway by biological transformation in a nitro aromatic compound.

The evidence from much research is that energetic nitroaromatic compounds can be transformed by microbial attack through two different pathways. One pathway is the reduction of the nitro group to the amino group, followed by an oxidative deamination to a phenol with the release of ammonia. The other pathway is by the release of a nitro group such as nitrite and the corresponding formation of a phenol (Kaplan 1990, McCormick et al. 1976). However, for the systems studied by Kaplan (1990), the nitrite release pathway did not appear significant. Yet, the research by Spanggord et al. (1991) on the aerobic biodegradation of DNT has proven this pathway to be tenable.

Usually, the first step in the biological reduction of a nitroaromatic compound is the conversion of the nitro

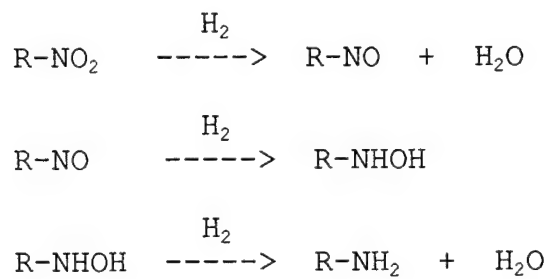


Figure 2.2

The Generic Pathway for the Biological Reductive
Transformation of a Nitro Aromatic Compound

[After Yamashina et al. 1954]

group to an amino group via the hydroxylamino intermediate transformation product (Channon et al. 1944, Funk et al. 1993). However, the hydroxylamino intermediates may also undergo reactions yielding complex azoxy compounds (Carpenter et al. 1978, Kaplan 1990, McCormick et al. 1976).

The order of the reduction rate of nitro compounds is consistent with the "electro-negativity rule." This rule says that the rate of reduction of nitro compounds increases with increasing electron withdrawing power of the groups at the *para* position (Shikata and Tachi 1938, Kaplan 1990). Therefore, the nitro reduction usually starts with the *para* nitro group (McCormick et al. 1976, Roberts et al. 1992) which is the most easily reduced. This results in monoaminonitrotoluene (MANT) isomers. The next reduction occurs at one of the *ortho* groups producing diaminonitrotoluene (DANT) isomers (Funk et al. 1993).

Under aerobic conditions the bacterial reduction of TNT stops at the DANT isomers. Apparently, the bacterial reduction of the third nitro group occurs only under strictly anaerobic conditions (Roberts et al. 1992, Funk et al. 1993). Therefore, depending upon the reducing potential of the system, one, two, or three of the nitro groups may be reduced to amino groups (McCormick et al. 1976).

Although the pH is not considered an important parameter in the adsorption phenomena (Pennington 1988, Pennington and Patrick 1990) it is important in the reduction of TNT. Funk et al. (1993) determined a near neutral pH optimum for reductive TNT metabolism. Further, the maintenance of a low to neutral pH minimized intermediate polymerization (Funk et al. 1993).

Numerous species of mammal, bacteria, fungi, and plants degrade TNT into various classes of chemical products. The transformation products resulting from the biological attack of these organisms will depend, to a large extent, on the mode of nitro reduction: aerobic or anaerobic.

Aerobic Environment

Although enzymes extracted from aquatic sediments reduce TNT to amino compounds (Wolfe et al. 1994, van Beelen 1994), the biological metabolism of TNT under aerobic conditions promotes the production of unstable hydroxylamino intermediates. These can react to result in the formation of azo or azoxy linkages with other intermediates and in turn to dimerization or polymerization (Channon et al. 1944, McCormick et al. 1976, Carpenter et al. 1978). Oxidation of aromatic amines can produce azoxy compounds (Smith 1965) as illustrated by the sequence shown in Figure 2.3.

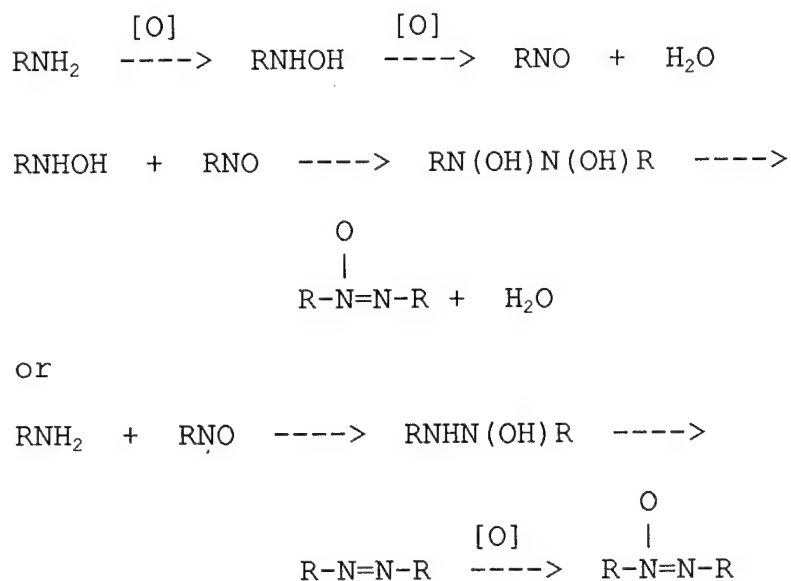


Figure 2.3

A Scheme Showing the Formation of Azoxy Compounds Produced
by Mild Oxidation of Aromatic Amines

[After Smith 1965]

Polymer formation results in a compound that is of a more complex nature than the original material. Also, it may be a common occurrence in the biotransformation of aromatic compounds (Carpenter et al. 1978). These complex products may be more stable and hazardous than the parent TNT material.

In any investigation of the fate of organic compounds subjected to biological treatment processes, the possibility of polymer formation must be considered. This is of particular concern when the polymers formed may be persistent in the environment and of unknown impact on ecosystems (Carpenter et al. 1978).

Anaerobic Environment

Metabolism of TNT under anaerobic conditions likely circumvents much of the aerobic polymerization problem, however, large amounts of precipitate have been observed from anaerobic TNT metabolism at pH 8 (Funk et al. 1993). The composition of this precipitate is unknown but may be formed of azoxy compounds. Still, under anaerobic conditions, there are fewer opportunities for the intermediates to form linkages because the reductions occur so rapidly that the hydroxylamino intermediates do not accumulate, plus, the reaction readily proceeds to the amino products (Roberts et al. 1992).

Anaerobic conditions are required for the enzymatic reduction of nitro to amino compounds because the hydroxylamino [R-NHOH] intermediates are reoxidized in air to the nitroso [R-NO] form (Kaplan 1990). Also, anaerobic conditions are preferred for the biotransformation of DNT, the major by-product of TNT manufacture (Liu et al. 1984).

Complete anaerobic degradation occurs in two stages. First, there is a reductive stage in which TNT is reduced to its amino derivatives. Then, there is an oxidative stage that continues the degradation of the amino compounds to nonaromatic products. This final stage can only begin after the reduction of the final nitro group (Funk et al. 1993). For actual biological degradation and not just product transformation, the reductive stage must be followed by the oxidative stage to yield TNT mineralization.

Anaerobic reduction is also strongly influenced by the strength of the reducing environment. Research conducted with different electron accepting conditions proves that significant TNT degradation can occur under nitrate reducing conditions by a bacterial consortium. Of the electron acceptors investigated (nitrate, sulfate, and carbon dioxide) a significant removal of TNT was observed in the enrichment culture using nitrate as the electron acceptor (Boopathy et al. 1993).

TNT Nitro Group Reduction

Most of the major products derived from TNT metabolism originate from the hydroxylamino compound (McCormick et al. 1976). Transformed amino aromatic compounds are the resulting products of this stepwise nitro group reduction of TNT (McCormick et al. 1976). A proposed pathway for the biotransformation of 2,4,6-trinitrotoluene by nitro group reduction is shown in Figure 2.4. Note that transformed compounds do not denote degradation, instead just superficial modifications.

According to present theory, formation of a diphenol is a prerequisite for the actual degradation of an aromatic nucleus (McCormick et al. 1976). Thus, the aromatic nucleus must carry at least two hydroxyl groups, *ortho* or *para* to each other for actual ring cleavage to occur (Dagley 1975). Although the nitro groups on the TNT molecule are reduced by both aerobic and anaerobic systems (Kaplan 1990, McCormick et al. 1976, Pereira et al. 1979), no evidence has been found for the formation of diphenols, thus no evidence for cleavage of the aromatic ring (Kaplan 1990).

TNT Photodecomposition

TNT is photochemically unstable. When in solution and exposed to sunlight it quickly degrades into a host of

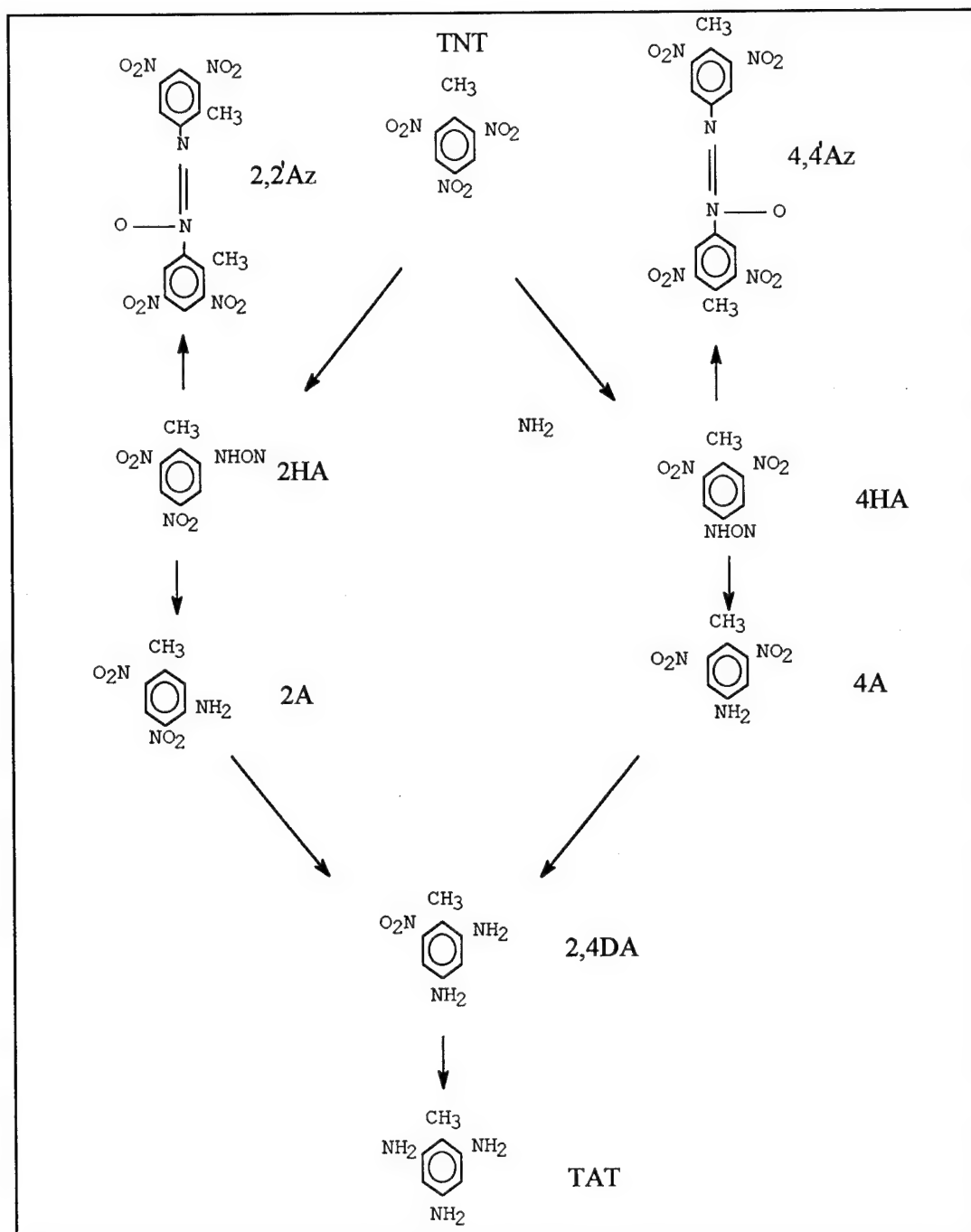


Figure 2.4

Proposed Transformation Pathway of TNT by Nitro Groups
Reduction [After McCormick et al. 1976]

photodegradation products; not all of them have been identified (Kaplan et al. 1975, Burlingson et al. 1979). Burlingson et al. (1979) did identify about half of the products resulting from an irradiated solution of TNT saturated distilled water. Table 2.3 lists the major photodegradation products identified in that research. Also included in the list is the expected percentage yield for each of the identified products. Two major photo-decomposition products, 2-Amino-4,6-Dinitrobenzoic Acid and 2,4,6-trinitrobenzaldehyde (TNBAL), accounted for approximately 20% of the identified photodegradation products (Burlingson 1980).

A proposed primary photo-degradation pathway is displayed in Figure 2.5 showing the TNT anion formation. All photo-products appear to result from the ortho-nitro group's intramolecular interaction with the methyl group. No para-position chemistry was observed (Burlingson et al. 1979, Suryanarayanan et al. 1972).

In experiments on aerated river water, the photodegradation process goes from 2,4,6-Trinitrotoluene through the 2,4,6-Trinitrobenzaldehyde then through some unknown complex product(s) to the 1,3,5-Trinitrobenzene (TNB) (Burlingson 1980). Because the photo-breakdown product TNB is very stable photochemically, its eventual disappearance, in Burlingson's experiment, was attributed to

Table 2.3

Photodegradation Products of an Irradiated TNT Distilled
Water Solution [After Burlingson et al. 1979]

Compound	Expected Yield
1,3,5-Trinitrobenzene (TNB)	0.5-1.0%
* 4,6-Dinitroanthranil	3.0-4.0%
* 2,4,6-Trinitrobenzaldehyde (TNBAL)	8.0-10%
* 2,4,6-Trinitrobenzyl alcohol	1%
3,5-Dinitrophenol	1%
2,2'-Dicarboxy-3,3',5,5'- tetranitroazoxybenzene, II	trace
2,2'-Dicarboxy-3,3',5,5'- tetranitroazobenzene, III	7%
2-Carboxy-3,3'-5,5'-tetranitro-NNO- azoxy benzene, IV	2%
2,4,6-Trinitrobenzoic Acid	1%
N-(2-Carboxy-3,5-dinitrophenyl)-2,4,6- trinitrobenzamide, V	1%
2-Amino-4,6-Dinitrobenzoic Acid, VI	11%

* Compounds identified as more photo-sensitive than TNT.

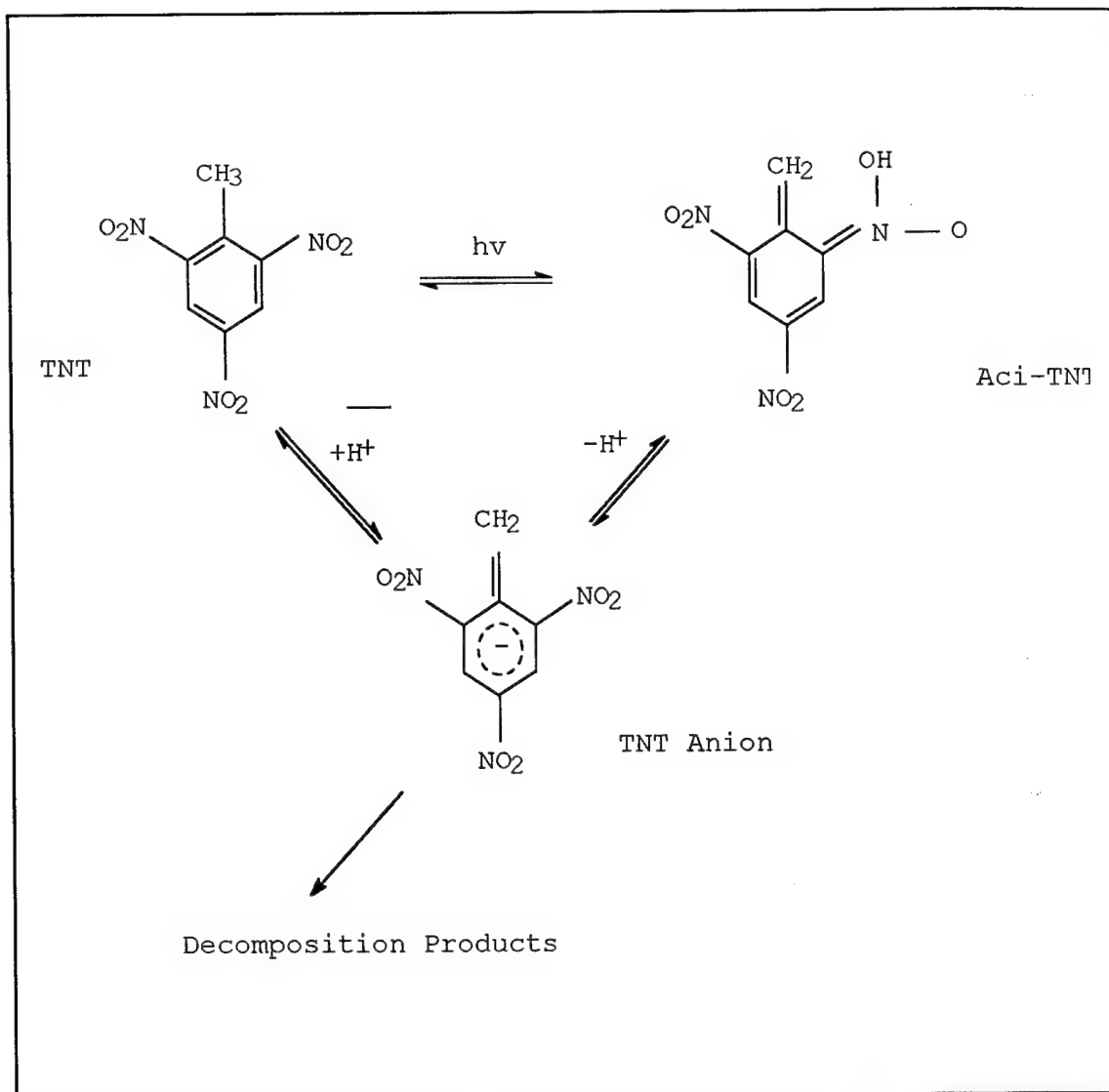


Figure 2.5

Proposed Primary Photo Chemistry Mechanism

[After Burlingson et al. 1979]

some biotransformation process. Consistent with this, a separate study has shown that the moderately halophilic obligate anaerobe eubacteria *Haloanaerobium praevalens* reduces nitrobenzene to aniline (Oren et al. 1991).

Reduction of an aromatic nitro group to the corresponding aniline is thought to occur by a series of electron addition and protonation steps (March 1985) where nitrosobenzene and phenylhydroxylamine are the intermediate as outlined in Figure 2.6.

The photo-breakdown process is very sensitive to pH with the rate of TNT disappearance inversely proportional to the acidity of the aqueous solution. Burlingson et al. (1979) shows pH values of 1.1, 7.4, and 11.1 result in the TNT decomposition percentages of 17.5, 50, and 95.9 respectively. Although pH does influence the amount of photo decomposition, its variation over the range 3.6 to 7.4 made little difference in the observed product composition. However, a significant color change occurred when the pH of the solution mixture increased from 7.4 to 8.5; the intensity of the photolysate color changed from red to burgundy.

Burlingson et al. (1979) also determined that the initial TNT concentration undergoing photolysis contributed little to the resulting product composition. They based

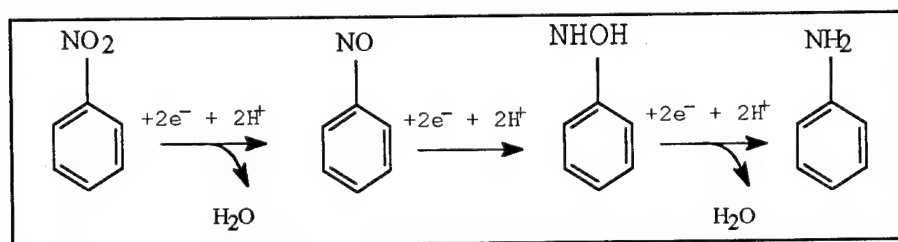


Figure 2.6

Reduction of an Aromatic Nitro Group to the Corresponding
Aniline Through the Nitrosobenzene and Phenylhydroxylamine

[After March 1985]

this determination on analyses of the photolytic decomposition of TNT solutions incorporating both a high and a low TNT concentration of 100 ppm and 4.5 ppm respectively.

Distilled water-TNT solutions were found to be very stable in the absence of light and under neutral to acidic conditions (Burlingson et al. 1979). Additionally, the major product resulting from the irradiation of a TNT-distilled water solution was identified as TNBAL. Thus, the major photo-generated product differed depending upon the water source: TNT-containing environmental waters formed TNB while TNT-distilled water formed TNBAL. Burlingson (1980) suggests that CO_3^{-2} or HCL^- could be the agent responsible for this difference in resulting product formation between the waters.

An insoluble reddish brown precipitate was also found to result from the photolysis of TNT (Burlingson et al. 1979). They suggest that this insoluble residue is composed of oligomers of azo and azoxy linked nitroaromatic rings. This suggestion is based on known chemistry and observations of the resulting photodecomposition products. They also found that several TNT decomposition products exhibited a photosensitive nature, some to a greater extent than the parent TNT (Burlingson et al. 1979).

TNT Product Photodecomposition

Besides studying TNT photo breakdown, Burlingson et al. (1979) studied several of the major TNT products for photodecomposition affects. They determined the photodecomposition rate for a 2,4DNT aqueous solution to be about an order of magnitude slower than that of TNT. They also identified seven of the resulting products from a 2,4DNT photolysis. These products were analogous to the TNT photodecomposition products. Also the resulting acidity profile was similar to that of TNT where the rate of 2,4DNT disappearance increased with increasing pH. However, the color change resulting from the reaction was significantly different; instead of a turbid brick-red for TNT, it was a turbid yellow-brown for the 2,4DNT.

Photolysis of another DNT isomer, 2,6DNT, yielded at least forty photodecomposition products most of which do not appear analogous to those of TNT (Burlingson et al. 1979). However, the rate of 2,6DNT disappearance again increased with increasing pH.

Burlingson et al. (1979) also investigated the photodecomposition of four aminodinitrotoluenes (ADNT) found in condensate wastewater. They reported that ADNTs are photo degraded at a much slower rate than the TNT. Identification of the resulting products were not made because of the long photolysis times required to generate a

sufficient quantity of individual products required for the determination. They did theorize that the ADNT photochemistry is similar to TNT, 2,4DNT, and 2,6DNT (Burlingson et al. 1979).

Terrestrial Plants

Many species of plants can uptake and metabolize TNT. Research with rooted terrestrial plants (yellow nutsedge - *Cyperus esculentus* L.) grown in hydroponic cultures containing TNT concentrations of 5, 10, and 20 mg/l shows TNT and its metabolites, 2ADNT and 4ADNT, were detected throughout the plants (Harvey et al. 1990, Palazzo and Leggett 1986). However, the plants experienced detrimental effects directly attributable to the TNT exposure. These adverse effects occurred rapidly and at solution concentrations of 5 mg/l and higher (Palazzo and Leggett 1986). Most conspicuous was the reduced plant growth in the root, leaves, and rhizomes. TNT exposure reduced total plant yields by 54 to 74% and root weights by 95% (Palazzo and Leggett 1986). Increasing the TNT concentrations in contact with the plants also increased the concentrations and quantities of both TNT and metabolites in the plants (Palazzo and Leggett 1986, Cataldo et al. 1989).

Plants that were studied favored the formation of 4ADNT over reduction to 2ADNT (Harvey et al. 1990, Palazzo and Leggett 1986). Also, Palazzo and Leggett (1986), proposed that the TNT metabolites were actually formed internal to the plant because they found measurable metabolites in analyses of plant tissue. This is logical as the TNT-culture solution was applied as a continuous feed with only TNT in solution.

Many experiments conducted with hydroponic systems show that TNT adsorptive capacity for plants is high. Total uptake into plant tissues is directly related to the concentration of TNT to which roots are exposed and results indicate that both TNT and ADNT are adsorbed.

Following root adsorption, greater than 75% of the accumulated amount was retained within the root. Analysis of acid-hydrolyzed and nonhydrolyzed tissue extracts suggests TNT-derived plant accumulation products are stored and transported as polar conjugates (Cataldo et al. 1989).

Degradation Resistant

Previous research shows that, in general, TNT is resistant to biodegradation by most bacteria and fungi (Fernando et al. 1990). Also, TNT breakdown in solution is

possible only when it is in low concentrations (Rogovskaya 1951).

As many TNT wastes have been buried, Osmon and Andrews (1978) investigated the degradation of TNT materials using a simulated sanitary landfill scenario. They found that for the soil environment studied, the microflora modified the TNT molecule into various conversion products without mineralization. Also, that the process was much too slow to be considered a viable treatment method and TNT-bearing materials should not be landfilled as a storage or treatment method.

Extensive research does not yield any evidence for the biological cleavage of the aromatic ring of TNT and the resulting degradation to CO_2 does not occur (Carpenter et al. 1978, Fernando et al. 1990, Kaplan and Kaplan 1983, Harvey et al. 1990). A major factor in the resistance of TNT to cleavage of the aromatic ring may be the resistance of the highly substituted molecule to enzymatic hydroxylation (Carpenter et al. 1978). However, another reason for the failure of TNT biotransformation products to be further biodegraded could be the formation of polyamides that are resistant to microbial action (Carpenter et al. 1978).

A pilot plant study has shown the ability of an aerated oxidation ditch facility to adequately biotransform TNT to

the amines. However, the process required the addition of a supplemented cornsteep water nutrient with bacterial floc and there were several operational drawbacks to overcome. The most significant obstacle was the initial low TNT concentration level necessary to keep the resulting toxic amines at an acceptable level in the effluent (Hoffsommer et al. 1978). Previous biological treatment systems for TNT-contaminated wastes have proved the resiliency of this compound to microbial degradation (Kaplan and Kaplan 1983).

TNT Contaminant Elimination

Over the years, the elimination of TNT contamination from the environment has been approached with a number of potential solutions. These endeavors have involved physical, chemical, and biological techniques. Physical avenues include activated carbon absorption, air stripping, filtration, and incineration. Chemical operations include solvent extraction, surfactant precipitation, and neutralization. Biological measures include denitrification and fermentation systems (Kaplan 1990).

Much has been learned about the fate of TNT exposed to these various strategies. Significantly, many of these techniques result in the generation of TNT-biotransformation products that exhibit higher mutagenicity potential than the

parent TNT (Funk et al. 1993, Kaplan and Kaplan 1982a, Kaplan 1990).

These methods, for the most part, have proven to be costly and inefficient (Won et al. 1974). Also, few were specifically designed to eliminate soil contamination. Current technology and emerging research ventures for contaminated soils remediation converge toward incineration, compost, extraction (surfactants), and various biological systems.

Incineration

Incineration is the current technology for remediation of TNT-contaminated soils. However, this treatment is very expensive; it costs approximately \$800 per ton (Funk et al. 1993). Further, the method is very ecologically destructive and poses possible health risks to workers because it requires the physical removal, transport, and storage of the contaminated soils (Boopathy and Kulpa 1992). Regardless of the associated problems, incineration is currently the only available means for remediation of significant quantities of TNT contaminated soil (Funk et al. 1993).

Composting

Composting is a biological process that produces organic and inorganic by-products and heat. Composting TNT

contaminated materials requires the mixing of contaminated materials with bulking materials and adding readily degradable organic components. Bulking materials are added to increase the porosity and aeration of the entire composite blend of materials (Williams et al. 1992). Biodegradable organic matter is needed to mediate the compost environment for microbial organisms.

Compost matrix is usually shaped into a pile or placed in a reaction vessel after materials addition to afford adequate heat retention and process control. Heat is an extremely important factor in the compost system by providing the necessary environment for an increased contaminant degradation rate. This rate increase results because the elevated temperatures increase both contaminate solubility and mass transfer potentials due to the increased biological requirements (Williams et al. 1992).

Composting is mainly an aerobic process and requires regular air exposure. However, anaerobic microenvironments likely form within the inmost portions of the compost medium (Williams et al. 1992). The actual inner environment may also be controlled by the technology used for the process: windrow, aerated static pile, or mechanical vessel.

In composting field tests, solvent extractable munitions concentrations were significantly reduced (Williams et al. 1992). Williams et al. (1992) describe the

mechanisms responsible for the reductions to be sorption of both contaminants and transformation products to the compost matrix. This results in the incorporation of munitions and related products into environmentally stable molecules such as humic acids and mineralization to carbon dioxide, water, and other inorganics (Williams et al. 1992).

By using ^{14}C labeled TNT, Kaplan and Kaplan (1982d) have investigated and proposed a biotransformation pathway for TNT in compost systems as outlined in Figure 2.7. Noticeably, no significant ^{14}C -labeled compounds, $^{14}\text{CO}_2$, or volatile amines were recovered in this study (Kaplan and Kaplan 1982d). Another compost research effort has shown that no aromatic nitro-bodies are lost to the environment as volatile materials during TNT compost degradation (Klausmeier et al. 1982). This suggests that TNT and TNT transformation products are nonvolatile in a composting operation.

The composting procedure is promising as a potential treatment for TNT contaminated soil remediation except that it requires long incubation times and is costly to set up and maintain (Funk et al. 1993). Also, it requires large quantities of additives thereby increasing the resulting mass of materials (Boopathy et al. 1993). Depending on the analysis of the final materials, these may require special measures for handling and disposal. Additionally, the

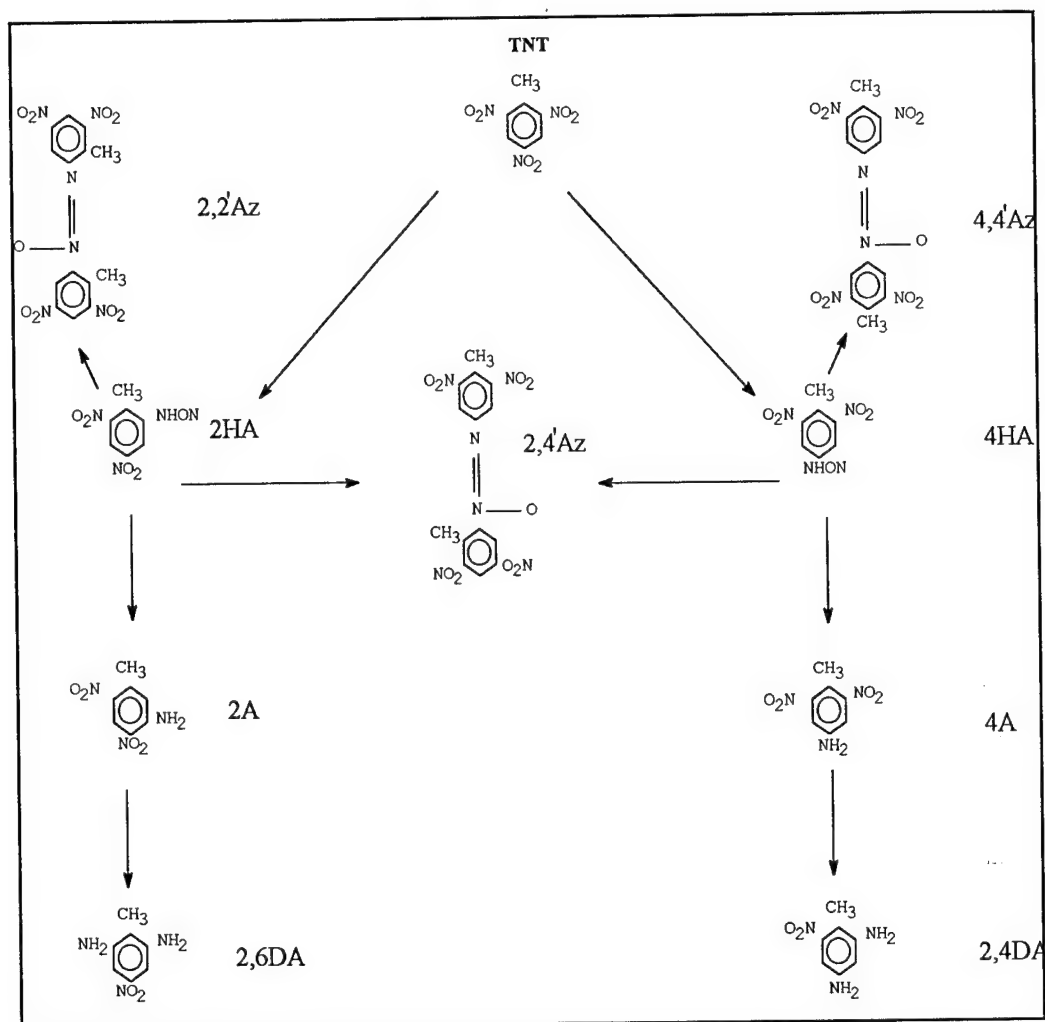


Figure 2.7

Proposed Biotransformation Pathway for TNT in Compost
Systems [After Kaplan and Kaplan 1982d]

interactions between TNT, TNT biotransformation products, and soil components may make the transformation products more stable and thus longer lived. Both, the 2ADNT and 4ADNT products have been identified in compost system studies (Kaplan and Kaplan 1982e, Kaplan 1990) and these water soluble amino compounds must also be tightly bound or eliminated.

Klausmeier et al. (1982) received a positive mutagenicity test; and more research was deemed required to ascertain the transformation products and toxicity of the final material (Kaplan and Kaplan 1982e). Once these determinations are accomplished, assuming nontoxicity of the final product, composting may prove competitive with incineration (Funk et al. 1993).

Extraction (Surfactant)

Using a surfactant as a binding treatment for TNT elimination has been researched for both contaminated wastewater and soil systems. First, surfactants were investigated as an inline wastewater treatment alternative to eliminate the TNT discharge to the environment. Certain amino surfactants under alkaline conditions rapidly complex TNT to form a water-insoluble, nonexplosive precipitate that can be separated from the effluent. Since studies revealed only traces of TNT in the wastewater after treatment and

filtration, the process was proposed for in situ treatment of contaminated soils (Kaplan and Kaplan 1982a).

Three surfactant treatments were tested in one study using soil columns: Duoquad T-50, Duomeen T, and Arquad T-50 (Kaplan and Kaplan 1982a). However, several problems were revealed. First, excessive amounts of surfactant were required for treatment of contaminated soils. Then, TNT breakthrough occurred on the soil columns before the contaminate levels dropped below detection limits. Finally, a significant amount of surfactant leached out of the column before breakthrough (Kaplan and Kaplan 1982a).

Another problem resulted from the excess sodium hydroxide required with the surfactant treatment (Kaplan and Kaplan 1982a). This component of the treatment elevated the solution pH to such a level that soil organic components were leached from the soil. This is a serious flaw in the method because it provides for both decreased soil quality and an increased potential for groundwater degradation.

Also, the 2A and the 4A TNT reduction products were not complexed and their concentrations were not significantly reduced by any of the surfactant treatment methods tested. This is another significant defect as these transformation products are very water soluble and hazardous, so failure to

eliminate or immobilize them suggests another potential for increased groundwater contamination (Kaplan and Kaplan 1982a).

Aqueous decomposition studies of the complexed materials show them to be unstable in water over long periods (Kaplan and Kaplan 1982a). Thus, depending on disposal methods, this process could worsen the contamination problem. The long term instability of the complexes did not appear biological in origin but no elaboration or conjectures were forthcoming from the authors (Kaplan and Kaplan 1982a).

Kaplan and Kaplan (1982c) have identified another major surfactant problem; the mutagenic properties were greater for the TNT-complexed materials. Mutation rates resulting from these were greater than the rate resulting from exposure to either TNT or the surfactant material alone (Kaplan and Kaplan 1982c). Soil burial of the complexed materials has been proposed but may not be a suitable method of disposal with the TNT-surfactant complexes presenting a greater hazard than the parent TNT (Kaplan and Kaplan 1982c).

Biological Systems

Biological remediation of TNT contamination has been and continues to be investigated. There appears to be a universal metabolism of TNT by bacteria, animal, and plant

systems to the amino isomers (Harvey et al. 1990). This reduction to the amino products is the greatest drawback for biological TNT reduction, as the amino isomers are toxic and mutagenic (Harvey et al. 1990) plus, TNT transformation does not mean elimination of the TNT associated problem.

TNT has also been reported to interfere with the normal biochemical oxygen demand (BOD) in sewage (Rogovskaya 1951). Ruchhoft et al. (1945) report that TNT concentrations above 1 mg/l have a retarding effect on sewage BOD. The authors conjecture that less oxygen is used because the TNT likely proves inhibitory or toxic to the microorganisms that would ordinarily use oxygen (Osmon and Klausmeier 1973).

However, there are many fungi, bacteria, and plants able to transform TNT (Roberts 1986). Screening of 190 fungi representing 98 genera showed that 183 had the ability to transform TNT. Yet, only 5 of this same group of 190 was able to transform DNT (Parrish 1977). Parrish (1977) did not recommend fungi application to TNT pollution abatement because of its inability to degrade the compound.

Gram-negative bacteria can also transform TNT. Some even grow well at TNT concentrations as high as 100 mg/l (Klausmeier et al. 1974). They have even been shown to use TNT as the sole source of carbon with some using TNT as the sole source of nitrogen for growth (Traxler 1974, Traxler et al. 1974). However, TNT concentrations over 50 mg/l have

been found inhibitory to gram-positive bacteria, actinomycetes, yeasts, and most fungi (Carpenter et al. 1978, Klausmeier et al. 1974). Of the many biological systems investigated for TNT remediation, some examples include:

Phanerochaete chrysosporium

Phanerochaete chrysosporium is often referred to as white rot fungus. It is a well-known lignin degrading (wood rotting) fungus, and has been the organism of choice for many investigators studying the remediation of hazardous wastes (Spiker et al. 1992). This fungus is one of the few microorganisms able to degrade lignin which is a naturally occurring and recalcitrant biopolymer (Bumpus 1989). The *P. chrysosporium* possesses nonspecific extracellular peroxidases enabling it to degrade the lignin (Spiker et al. 1992). These nonspecific enzymes also enable the organism to degrade other recalcitrant compounds (Bumpus et al. 1985).

Unfortunately, even small amounts of the contaminated soil from a representative contaminated munitions-processing site proved completely inhibitory to the fungus (Spiker et al. 1992). This fungus has also been found to be very sensitive to pure TNT (Spiker et al. 1992), therefore, *P.*

chrysosporium is probably not a good candidate for bioremediation of contaminated sites containing high TNT concentrations due to its exhibited TNT sensitivity (Funk et al. 1993, Spiker et al. 1992).

Pseudomonas

The bacteria *Pseudomonads* have and continue to be involved in TNT remediation studies. They are the predominant microorganisms in soil and water systems and are known to metabolically oxidize TNT (Osmon and Klausmeier 1973, Schackmann and Muller 1991, Won et al. 1974) but, only in the presence of other organic nutrients (Carpenter et al. 1978, Osmon and Klausmeier 1973). However, the resulting products from the aerobic transformations are considered as bad, if not worse, than the parent TNT.

TNT oxidation by these bacteria yield intermediate products such as: 2ADNT, 4ADNT, 2,2',6,6'-tetranitro-4-azoxytoluene (4Az), 2,2',4,4'-tetranitro-6-azoxytoluene (6Az), 4,6-DN-2-AT (2Am), 2,6-DN-4-hydroxyl-aminotoluene (OH-Am) and nitrodiaminotoluene (DiAm) (Won and Heckly 1974). The organisms were incapable of oxidizing the intermediates 2-Am and DiAm (Won et al. 1974). These results concur with the oxidation of TNT in other biological systems (Duque et al. 1993).

Recent investigations reveal a pseudomonas isolate capable of the complete biodegradation of DNT. Apparently the DNT serves as a carbon source (Spanggord et al. 1991). The initial attack involves the displacement of the 4-nitro group by molecular oxygen through dioxygenase canalization (Spanggord et al. 1991).

Desulfovibrio

Other recent literature reports that a sulfate reducing bacterium *Desulfovibrio* sp. (B Strain) isolated from a continuous anaerobic digester has successfully utilized TNT as a sole source of nitrogen. A proposed pathway for the metabolic transformation by this bacterium is shown as Figure 2.8 (Boopathy and Kulpa 1992).

The main intermediate observed was DANT and the process carried through to the triaminotoluene (TAT) (Boopathy and Kulpa 1992). Subsequent reductions, under nitrogen limiting conditions, resulted in TAT deaminated to toluene and in the process the isolate used the ammonium released from the original TNT as a nitrogen source for growth (Boopathy and Kulpa 1992). However, under nitrogen-rich conditions, i.e., in the presence of ammonium, TNT was converted to DANT and the reaction stopped. The investigators believe that this system could be used together with toluene degrading,

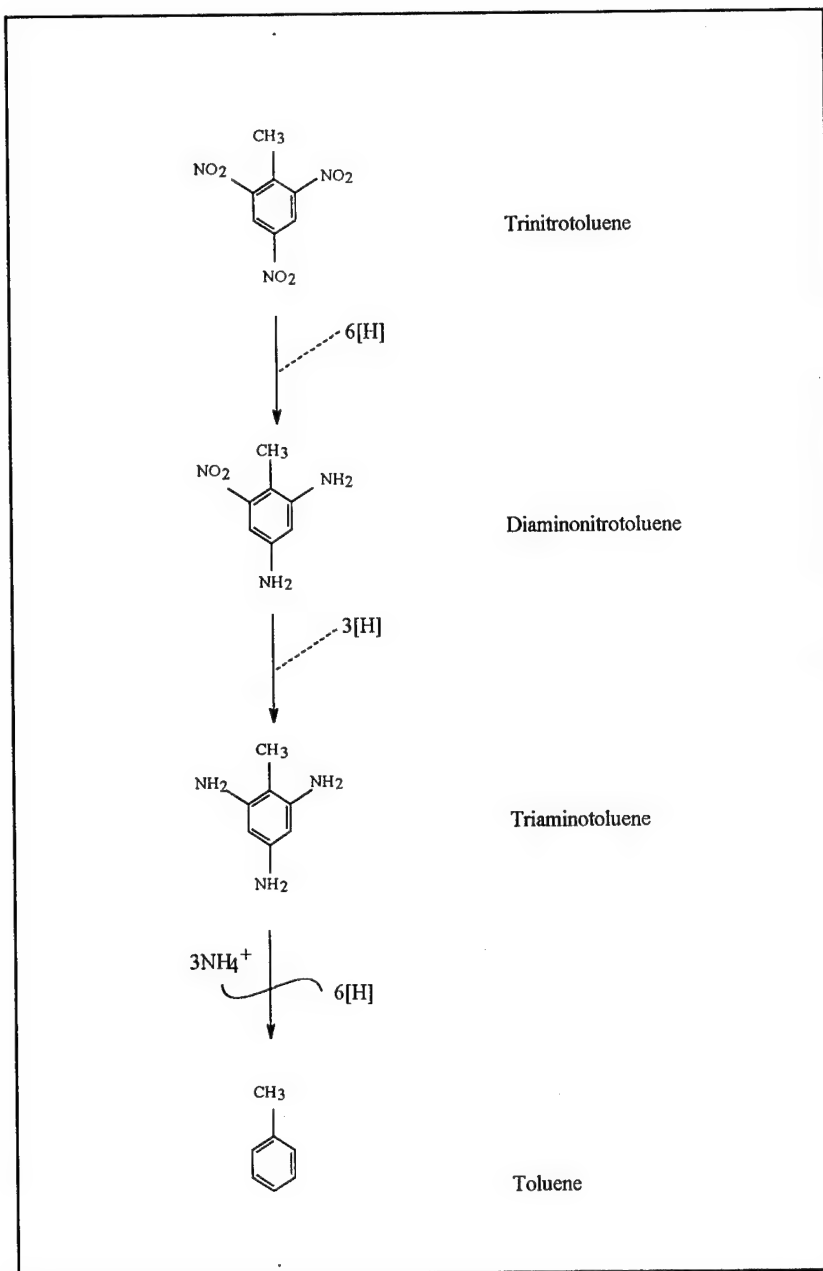


Figure 2.8
Proposed Anaerobic TNT Transformation Pathway by
Desulfovibrio
[After Boopathy and Kulpa 1992]

denitrifying bacteria, like *Pseudomonas*, to take the TNT degradation through toluene to CO₂ (Boopathy and Kulpa 1992).

Veillonella alkalescens

Cell free extracts of the strict anaerobe *Veillonella alkalescens* catalyzed the reduction, by hydrogen gas, of the nitro groups of many nitroaromatic compounds to the corresponding amino compounds (McCormick et al. 1976). The nitroreductase activity of *V. alkalescens* extracts were associated with protein fractions, one having some ferredoxin-like properties and the other possessing hydrogenase activity. The elimination of the hydrogenase from the reaction was not successful and it is unknown if the ferredoxin acts as a nonspecific reductase for nitroaromatic compounds (McCormick et al. 1976).

Nitella sp.

Research at the ERL-A identifies the aquatic plant *Nitella sp.* (Stonewort) proficient in reducing TNT to the corresponding amino transformation compounds (Wolfe et al. 1994). In laboratory experiments incorporating the Stonewort, the nitroreductase activity in the aqueous phase was high enough to reduce the TNT as fast as it was

solubilized (Wolfe et al. 1994). Of major significance, the dinitroamino and diaminonitro isomers were reduced as well (Wolfe et al. 1994).

The TAT product was identified but quickly disappeared; no TAT was detectable by day 43. TAT formation and disappearance was measured by micellar electrokinetic capillary electrophoresis (MECE) with a detection limit of 0.01 mg/l. It is theorized that the TAT was rapidly oxidized by autoxidation or by laccase activity to ring opened products that can be incorporated into the plant tissue or subsequently degraded to innocuous compounds (Wolfe et al. 1994).

A reductive transformation pathway was proposed from the data analyzed from the studies with Stonewort. It is shown in Figure 2.9 (Wolfe et al. 1994).

Analytical Measurements

TNT and TNT metabolites have been qualified by many different methods as annotated by Castorina (1980) and will not be exhaustively reviewed here. Only those of significance to this research will be outlined; most recent TNT quantification was accomplished by High Pressure Liquid Chromatography (HPLC).

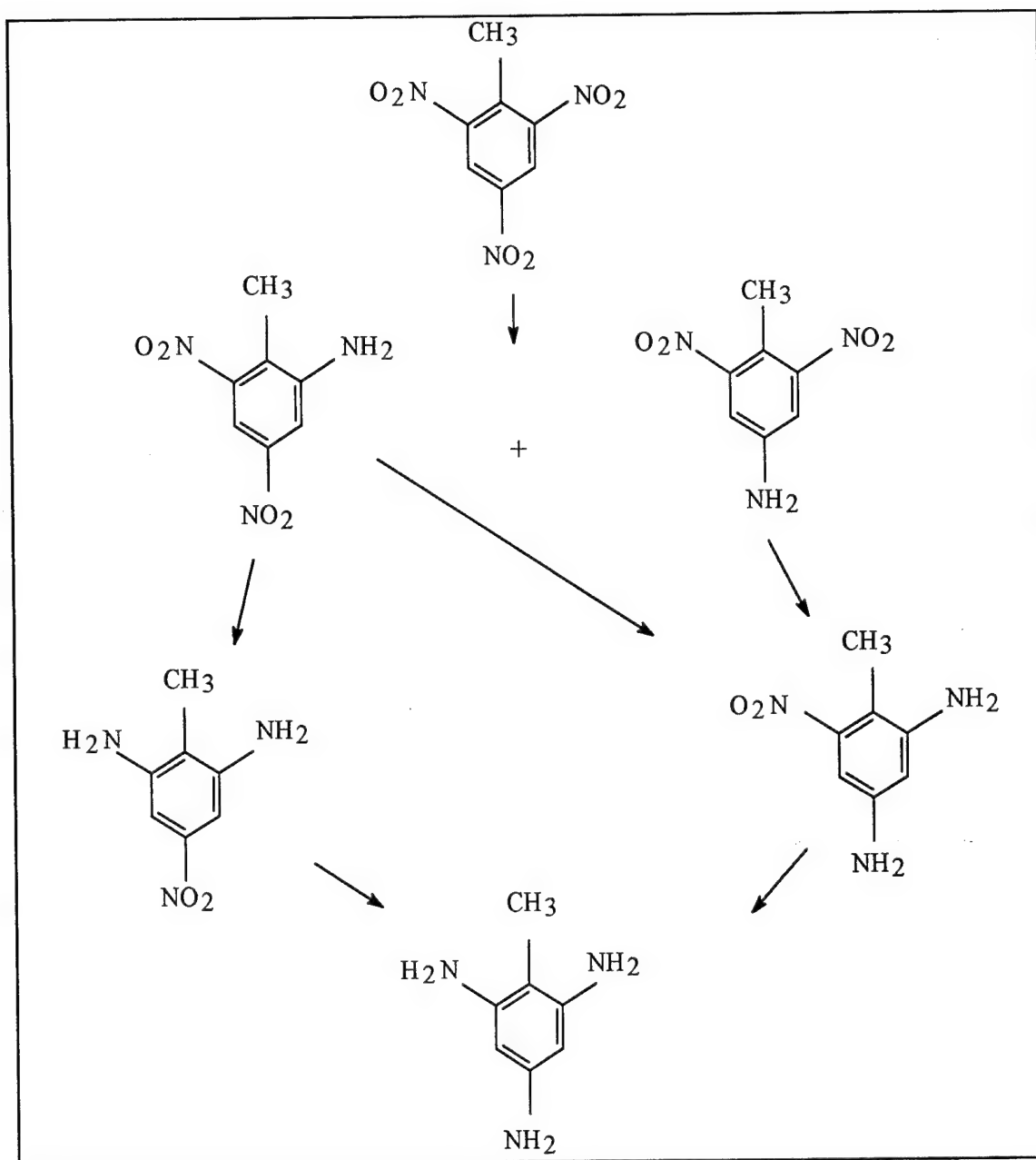


Figure 2.9

Proposed Mechanism of TNT Reduction with Extracted Sediment

Enzyme and Aquatic Plant Stonewort

[After Wolfe et al. 1994]

HPLC

Achievement of an adequate mass balance is crucial to successful experimentation. Harvey et al. (1990), using the HPLC, achieved a mass balance of over 88% for the TNT, 2ADNT, and 4ADNT compounds. Their major system components consisted of: a octadecyl silica column, an acetonitrile-water solvent gradient from 40% to 100% acetonitrile, and UV adsorption detection at 254 nm.

Similarly, sample components and treatment products must be identified before relationships can be established. Many researcher have used the HPLC to determine the nitro-organic components in munitions wastewater (Bauer et al. 1986, Jenkins et al. 1986). Their major system components consisted of: an LC-8 reverse phase column, a water-methanol-acetonitrile solvent constant 1.5 ml/min flow rate, and UV adsorption detection at 254 nm.

Additionally, the UV spectral profiles for TNT and its metabolites have been detected and quantified by an HPLC using diode array detection (Wolfe et al. 1994). This multiple wavelength analysis provides a profile map of the selected elute peak and can be compared to standards for additional confirmation of product identification.

Recently, Wolfe et al. (1994) have achieved good separation of TNT, monoamino, and diamino products by HPLC. Their major system components consisted of: a Hamilton

reverse phase column, a water-acetonitrile solvent constant 1.0 ml/min flow rate, and UV adsorption detection at 238 nm. Although the products were adequately separated, they were unable to adequately separate the product isomers with a constant solvent flow and they found that the triamino product eludes with the solvent front.

MECE

The limitations of the HPLC, regarding product isomer separation, are overcome by the micellar electrokinetic capillary electrophoresis (MECE) method. Wolfe et al. (1994) report superior product separation using this method including TAT isolation from the solvent front. In a separate study, Northrop et al. (1991) report both rapid and efficient separation and detection of many organic constituents from gunshot and explosives residues with the MECE. Still, product identification requires that standards of known concentration must be analyzed and compared to elute plots.

GC/MS

The Gas Chromatograph/Mass Spectrophotometer (GC/MS) is a better method for product identification. Once a particular TNT/TNT-product containing sample has been

concentrated, identification of the resulting elute peaks is usually straightforward (Ou et al. 1994b).

Standard Compound

For most analytical methods, to identify and/or quantify an unknown compound it must be compared to high purity standards of known concentration. Sitzmann (1974) details the preparation of eight initial TNT reduction products. However, most standards are easily purchased off-the-shelf and their generation by the researcher is seldom required.

Summary

Much effort and research have been invested in the elimination of TNT and TNT transformation products from the environment. Most of the past efforts were directed at: reducing effluent and attenuating increases in environmental contamination, quantifying the location and severity of the contamination problem, and estimating the bio-hazards associated with the contamination. In recent years, with the location and severity of the contamination better known, the emphasis has turned to remediation of these contaminated sites.

However, several major problems must be addressed and solved before environmental restoration can be applied in enough quantity to significantly reduce the current and future hazards associated with environmental TNT contamination. These include: reducing high remediation costs, developing a method capable of providing adequate cleanup in an acceptable time, and determining the degree of environmental restoration that is deemed acceptable. The selected method must prove robust enough for use at typically high TNT concentration contaminated sites. So, more studies must use real world conditions because remediation studies are seldom conducted with actual contaminated soil or at concentration levels found at many sites. Much of the research has been performed with a synthetic medium impregnated with pure TNT.

All these issues must be adequately addressed in the method selected for TNT soil contamination remediation. Further, the method must insure that the contamination cycle be broken, i.e., the intermediate products must not contaminate another medium and the generated products must prove nontoxic. It must therefore produce complete degradation which includes aromatic ring cleavage and not just simple TNT transformation.

Biological remediation has the ability to meet all these requirements although most major research efforts

involving these systems have met with only limited success. Just recently, however, bacteria have been discovered that might prove instrumental in future TNT remediation schemes.

Also, the significant TNT transformation abilities of higher or vascular plants have been mostly overlooked. Most plant studies have been concerned more with the contamination fate within the plant and possible food-chain introduction than with actual remediation of contaminated sites.

Research with the plant Stonewort evidences a potential TNT treatment opportunity using plants. Further, use of plants increases the prospect for in situ remediation.

CHAPTER 3

ANALYTICAL MATERIALS AND METHODS

There are many variables in any research venture. Also, there are many pathways to accomplish a specific research objective and interpretation of experimental results are often shaded by knowledge of these variables and pathways. This chapter details the specific materials and methods used throughout this research.

Chemicals and Standards

All TNT extractions, dilutions, and HPLC analyses were made with acetonitrile rated as HPLC grade and purchased from Fisher Scientific Supply, 711 Forves Ave. Pittsburgh, PA 15219. Another component of the HPLC solvent stream was a deionized water buffered to pH 10 with NaOH. Selected liquids were also pumped through the HPLC system at a one ml/min flow rate for a one hour duration to clean and maintain the column and piping system. These liquids were: buffered deionized water, methanol, acetonitrile, and a 5% nitric acid solution.

Both pH 4 and pH 7 buffer solutions were purchased from Fisher Scientific Supply for pH meter calibration and Eh probe certification. Quinhydrone, also for Eh probe certification, was purchased from Fisher Scientific Supply and acquired from Dr. Kathryn Flynn of the Auburn University (AU) Extension Forestry Department. Scintillation cocktail used in the radiochromatography analyses at the ERL-A was Ultima Gold LSC Cocktail by Packard.

TNT standards for comparison analyses were prepared from a 2,4,6-TNT base purchased from Chem Service, West Chester, Pa, and obtained via the ERL-A. The TNT reduction product standard materials were purchased from Accu-Stds and obtained from the ERL-A. Also, the labeled material (2,4,6-[ring ^{14}C]-trinitrotoluene) with a specific activity of 21.58 mCi/mM, was purchased from Chem Syn Science Lab, Lenexa, Kansas and obtained from the ERL-A.

Analytical Methods

HPLC analyses were used primarily to quantify TNT concentrations. Most MECE analyses, performed at the ERL-A, were mainly for product quantification and identification. Similarly, GC/MS analyses performed at the ERL-A were mainly for product identification.

High Pressure Liquid Chromatography (HPLC)

TNT concentration determinations were made by reverse phase HPLC analysis on a Varian 654 Data system connected through a local area network to a Varian 5560 HPLC using the Varian UV-200 UV-Vis Detector and a 9090 Varian Autosampler. The analytical reverse phase column was a Hamilton PRP-1 connected in line with the Hamilton PRP Guard Column. System conditions for analysis included a solvent stream composition of acetonitrile and buffered deionized water in the proportions of 65% and 35% respectively. Also, a uniform solvent flow of one ml/min and detection by UV absorption at 238 nm with the detector sensitivity set to 0.01 absorbance units per millivolt of output. These variable HPLC parameters for TNT concentration determinations were patterned after those being used at the ERL-A for similar research (Ou 1994a).

TNT and reduction products peak areas were integrated by the data system with hard copy output on a NEC dot matrix printer. Two HPLC sample loops were used over the course of the study. A 10 μ l loop was employed for the first field experiment analyses but was changed to a 50 μ l loop to improve TNT detection sensitivity for the remainder of the analyses.

¹⁴C Radiochromatography

Both combustion oxidizer, Packard model 307, and the Beckman LS6000LL scintillation counter were used at the ERL-A to quantify ¹⁴C components. Labeled product separations were made on a Waters 740 data module connected to an HPLC equipped with a Spectroflow 757 variable wavelength UV absorbance detector. The column used was the PRP-1 250 x 4.1 mm with a mobile phase flow of 1 ml/min and composition of 65% Acetonitrile and 35% deionized water adjusted to pH 10 with NaOH.

Gas Chromatography/Mass Spectrometry (GC/MS)

GC/MS analyses were performed on selected samples to confirm HPLC and/or MECE identified compounds by an analytical method developed at the ERL-A (Ou et al. 1994b). The analysis used a selective ion method and the HP 5890 series 2 GC interfaced with a HP 5971 MS (Cipollone 1994).

Micellar Electrokinetic Capillary Electrophoresis (MECE)

MECE analyses were made by a Spectra Phoresis 1000 system with a UV detector at the ERL-A (Ou 1994b). System conditions were: fused silica 70 cm x 75 μ m (i.d.) column; 25 mM sodium dodecyl sulfate (SDS) in 2.5 mM borate buffer (pH 8.56) mobile phase; hydrodynamic, usually two seconds (5.37 nL/s)m and 220 nm UV wavelength. Also, the UV

spectrum of selected peaks was obtained from the fast scanning detection (Ou 1994b).

Analytical Procedures

HPLC Analysis for TNT

At the beginning of each sample analysis session, the chromatographic area was determined for a series of samples containing known TNT concentrations. It was important to determine the peak retention time and peak area of these TNT standard samples of known concentration for each sampling session because small changes in the HPLC system affected the output chromatogram. A simple linear TNT concentration-chromatographic area plot was constructed through least squares analysis from the resulting standards data by the Borland Quattro Pro program. The least squares coefficient of determination, r^2 , for the linear data was normally greater than 90% for the standards plot. A representative example is shown as Figure 3.1.

After the standards plot was constructed, each experimental sample chromatogram made during that HPLC session was analyzed. If the sample contained TNT, there

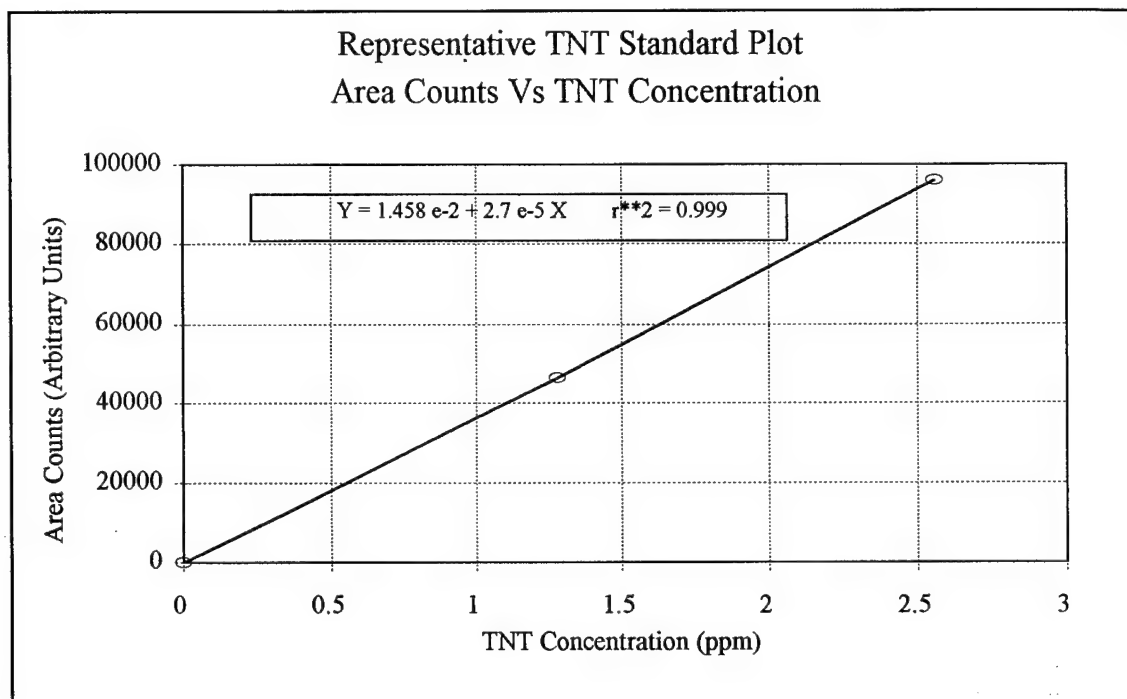


Figure 3.1

A Representative TNT Standards Plot

was an eluted peak at the retention time of the TNT standard and the area of this eluted peak, measured in area counts, was directly proportional to the TNT concentration of that sample. When the area counts that were generated for each sample were matched to the linear plot of the standards, the unknown concentration became readily evident. All samples were diluted with acetonitrile to yield elute peaks within the linear portion of the HPLC detection system.

HPLC Example Chromatogram

An HPLC chromatogram with the accompanying integrated area counts tabulated by time of elution was generated for each sample analyzed. These data are too bulky and numerous for inclusion, however, two HPLC-generated chromatograms are presented as examples. Also, the TNT concentration data deduced from pertinent chromatograms into numerical values are included in accompanying tables and charts in the appropriate chapters.

The first example chromatogram resulted from the HPLC analysis of a 1.28 ppm TNT standard; it includes peak retention times and peak area counts as shown in Figure 3.2 and is annotated to identify the TNT area counts. Another example chromatogram typifying the analysis of an actual TNT contaminated sample taken during this research is included as Figure 3.3. It is annotated to identify the area counts

CHART SPEED 0.2 CM/MIN
ATTEN: 16 ZERO: 5% 5 MIN/TICK



CHANNEL: 1A - 1 TITLE: 10:11 26 DEC 94
SAMPLE: 1.28 TNT METHOD: TNT CALCULATION: A% - ANALYS - OP

PEAK NO	PEAK NAME	RESULT AREA%	TIME (MIN)	AREA COUNTS	SEP CODE
1		3.2334	1.075	1715	BV
2		1.6667	1.287	884	VB
3		5.6052	1.576	2973	BV
4		5.2206	1.679	2769	VB
5		84.2741	6.324	44699	BB ==> TNT
TOTALS:		100.0000		53040	

Figure 3.2

Example HPLC Chromatogram for a 1.28 ppm TNT Standard
Concentration

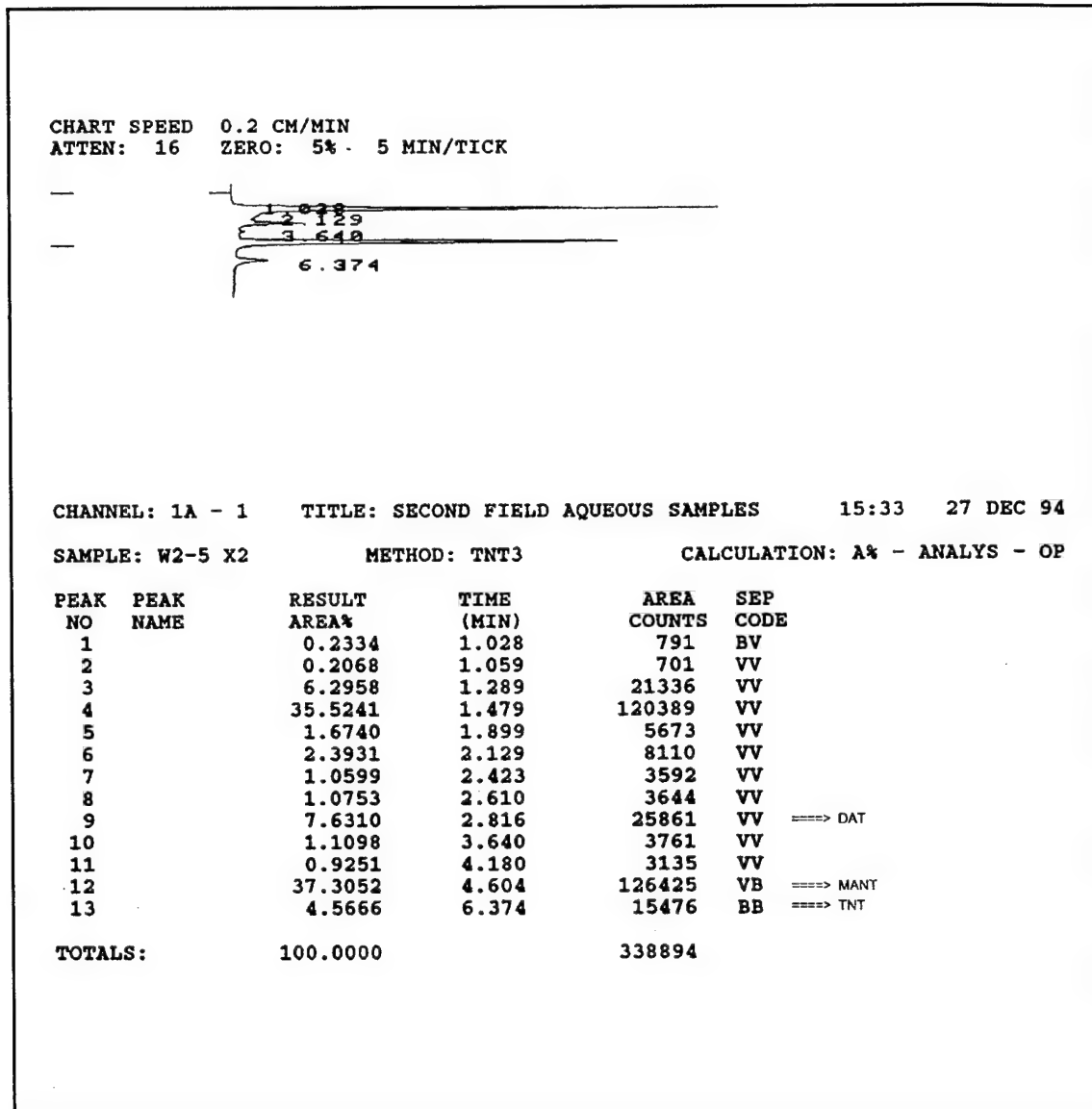


Figure 3.3

Example HPLC Chromatogram of an Actual Sample Showing Peaks, Retention Times, and Annotations for TNT, MANT, and DAT

resulting from the TNT and its major reduction products MANT and DAT.

¹⁴C Radiochromatographic Analysis

Radiochromatographic detection provided the means to quantify the mass balance of a ¹⁴C labeled TNT as it was transformed into labeled reduction products. This part of the research was performed at the ERL-A under the guidance of Dr. Tse-Yuan Ou.

During specific HPLC chromatographic runs, the column elute was collected at 20 second intervals for 15 minutes and then at one minute intervals for another 15 minutes. Each of these fractions were subsequently assayed for radioactivity by liquid scintillation spectrophotometry. Radiochromatograms were then generated from this data by plotting the disintegrations per minute (dpm) in each fraction over time.

Additionally, transformation products were collected from repetitive HPLC runs to accumulate enough material for subsequent mass-spectral identification by the GC/MS.

GC/MS

An example GC/MS chromatogram is included as Figure 3.4. It was generated from a sample composed of TNT and amino reduction product standards and is annotated to show

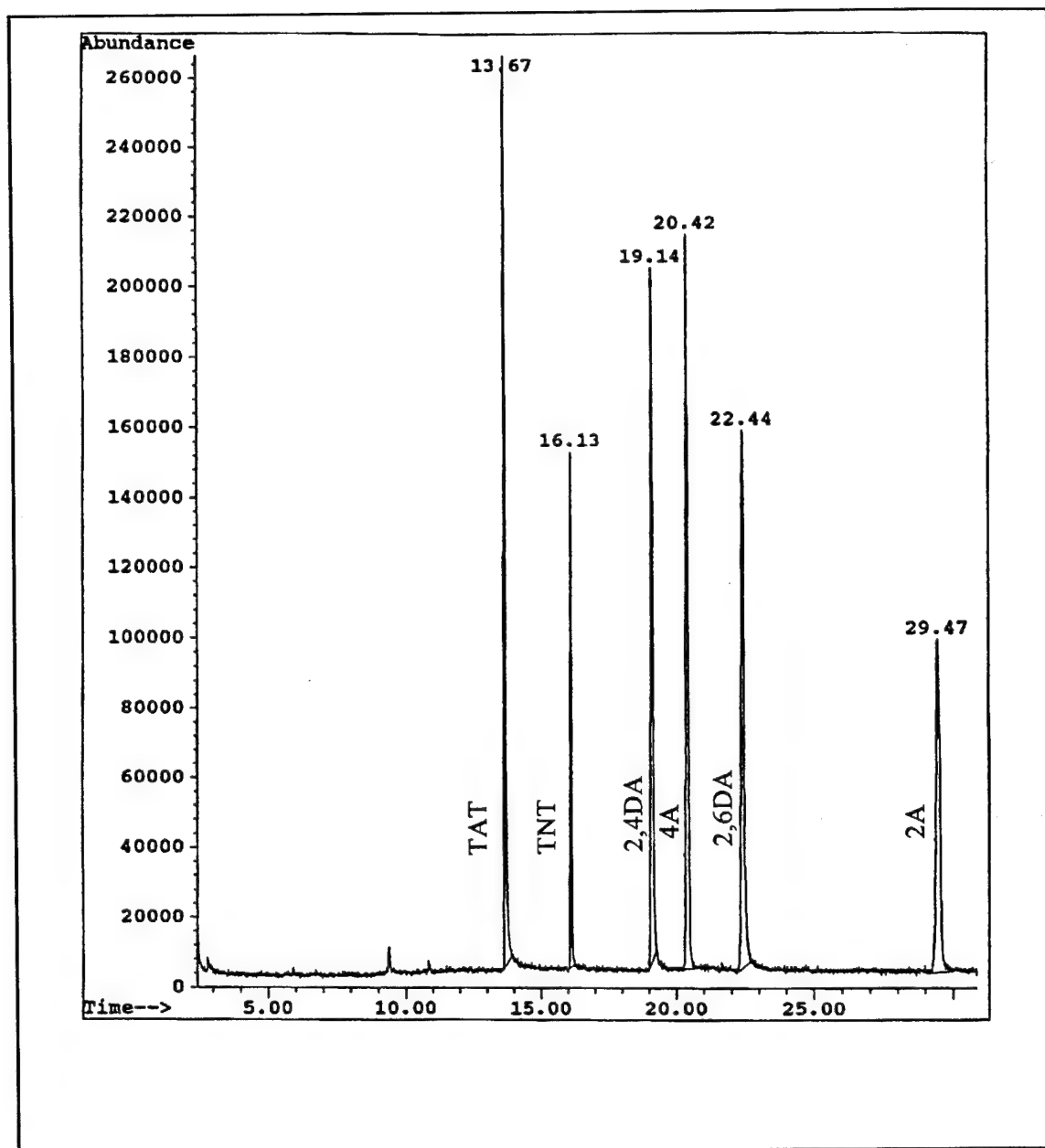


Figure 3.4

GC/MS Example Chromatogram showing the TNT and Amino
Reduction Product Standards

the retention time and identity of each peak (Ou 1994a). As most reduction products occurring within individual samples were below GC/MS detection limits, samples were concentrated for GC/MS analysis. Two methods for degradation products concentration were employed: elute collection from repetitive HPLC runs and the use of a Sep-Pak C-18 cartridge. As the repetitive collection is self explanatory, only the Sep-Pak method is detailed.

The steps involved in the Sep-Pak method include: condition cartridge, pass sample through cartridge, apply solvent to remove products from cartridge, and reduce volume of the resulting solvent-product containing sample. The initial conditioning step consisted of a six ml acetonitrile wash followed by another six ml wash with deionized water. After conditioning, a five to ten ml sample solution was applied to the cartridge, depending upon the concentration levels in the sample and the amount of available sample. Enough pressure was applied to the plunger of a 10 ml glass syringe containing the sample to generate a sample flow rate of 1 to 1.5 ml/min through the cartridge. This was followed by a two ml deionized water wash to flush residual sample from the cartridge. Then, two ml of acetonitrile was applied to the cartridge at about a 1 ml/min flow rate to remove the concentrated products from the cartridge.

Concentration was further enhanced by evaporation of this two ml products containing sample to 0.5 milliliter.

MECE Analysis

The MECE was also used for TNT and reduction product quantification. It proved a superior method by providing MANT and DANT isomer separation that can only be obtained on the HPLC by gradient programming. Also, the MECE method provided adequate TAT separation which eludes with the solvent front on the HPLC.

MECE Example Chromatogram

An MECE chromatogram was generated for samples from both field experiments. Only two of these chromatograms are included as general examples although the data obtained from the chromatograms are discussed in the appropriate data analysis sections. The first example MECE chromatogram in Figure 3.5 results from a sample of TNT and amino reduction product standards. Each peak is annotated to indicate the compound and each retention time is also marked. The second example chromatogram is from the analysis of an actual water sample from the "with plants" treatment taken the first week in field experiment two. It is shown as Figure 3.6 and the identified peaks are also annotated.

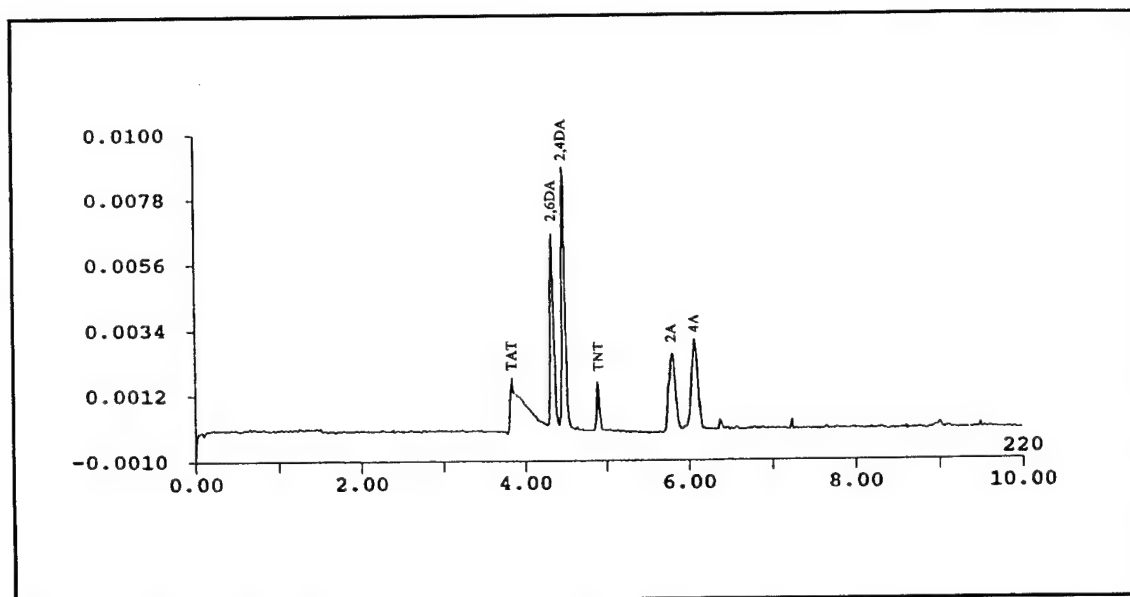


Figure 3.5

Example MECE Chromatogram of TNT and the Reduction Product
Standards

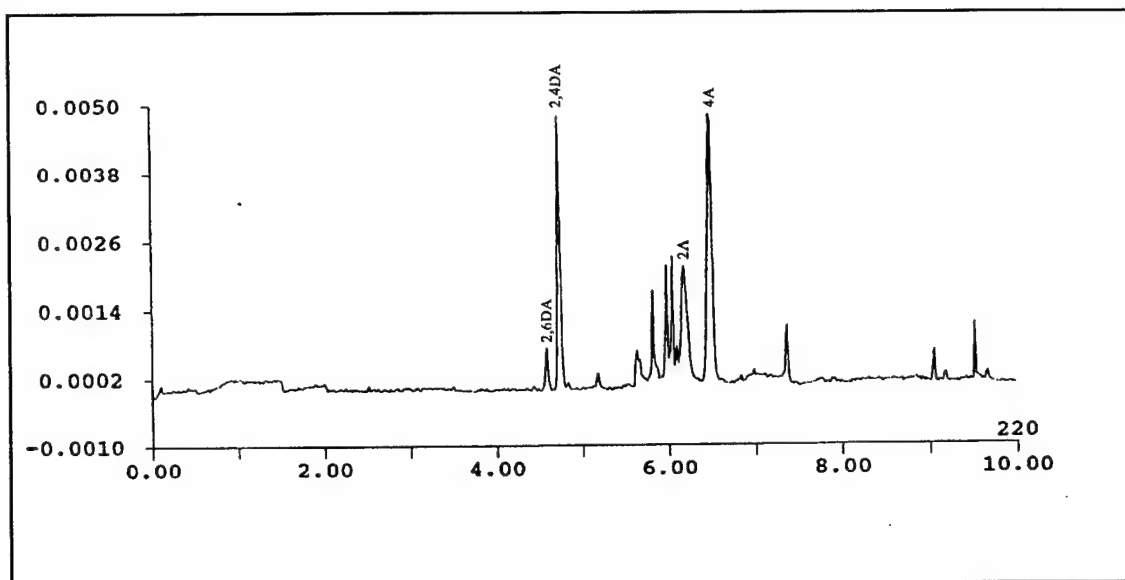


Figure 3.6

Example MECE Chromatogram of an Actual Water Sample Taken
From the "With Plants" Treatment in the Second Field
Experiment

UV Spectra of TNT and Reduction Products

UV spectral profiles for TNT and the amino reduction products have been determined by application of the MECE (Ou et al. 1994a). For each peak detected by the MECE, a unique profile can be generated to show the detection across a wide UV spectrum. This unique spectral data can be compared to known spectra of TNT and TNT reduction products standards as additional confirmation of the products previously identified by retention time alone.

Field Experiment Aqueous Phase pH Determinations

The pH of the water in the soil solution containers were measured in the field. Meter calibration was verified with a pH 7 buffer solution purchased from Fisher Scientific Supply before the field measurements were taken. All pH field measurements were made by a Fisher Scientific Accumet Mini-pH Model 955 portable pH/millivolt meter with a standard pH probe, model 13-620-108.

Soil Eh Field Determinations

Platinum field probes were constructed as specified by Faulkner et al. (1989) and used in the second field experiment. Each probe was made of a 1.3 cm segment of 18 gauge platinum wire soldered to a 12 gauge insulated copper wire, approximately 18 inches in length. Each solder weld

was epoxy sealed and covered by a small section of heat-shrink plastic tubing. Afterwards the solder rod shaft was used as a heat source to shrink the tubing before the final epoxy seal was applied to complete probe construction.

All probes were quality control checked with two quinhydrone and buffer solutions of pH 4 and pH 7 as detailed by Bohn (1971). Before the probes were installed in the field containers, they were thoroughly cleaned with distilled water and lightly sandpapered to a bright finish. After installation, a one hour equilibration period was observed before Eh readings were taken. Readings were made by a Fisher Scientific Accumet Mini-pH Model 955 portable pH/millivolt meter set to the mV setting and using a saturated calomel reference electrode purchased from Fisher Scientific, catalog number 13-620-51.

Statistical Determinations

Statistical evaluation of the soil TNT concentration data was performed by the SAS for Windows Version 8 computer program. The simple linear least squares determinations for TNT standards and other less complicated sample analyses were performed by Quattro Pro for Windows Version 5.

General Sample Preparation Procedures

Aqueous and solid phase samples required preparation before the TNT concentration could be measured on the HPLC. Each sample was filtered or centrifuged to settle suspended materials in both water and TNT-solvent samples. This was necessary to preclude clogging of the HPLC analysis system and to eliminate the variability introduced by TNT-containing particulate in suspension. In addition to requiring particulate removal, soil samples required both drying and extraction. Drying was necessary to reduce sampling errors introduced by excess moisture and extraction was required to reposition the TNT from a solid to a liquid state in order to perform HPLC concentration determinations.

Aqueous Phase Sample Preparations

Two procedures were employed for aqueous phase sample preparations. First, aqueous samples were filtered through a Whatman 0.2 μm nylon syringe filter to eliminate suspensions from the solution and present a filtered sample. However, due to the apprehension that some breakdown amino components might also be removed by this filtration, the filter process was discarded in favor of centrifugation. For centrifugation, each aqueous sample was placed in a 1.5 ml plastic centrifuge vial and centrifuged on a (Brinkman)

Eppendorf 5415 centrifuge at 14,000 rpms for 10 to 15 minutes. Sample aliquots for subsequent dilution were then removed from the clarified solution by a pipette; disposable plastic tips were purchased from Fisher Scientific Supply.

Solid Phase Sample Preparations

TNT must be removed from the soil matrix and be in solution in order to be quantified by HPLC analysis. Thus, each soil sample from the field experiment was dried, a representative subsample was removed, and then TNT was extracted from the subsample to present a sample suitable for HPLC analysis.

Sample Dilution for HPLC Analysis

Standard dilution techniques were used to lower the TNT concentration contained in samples into the required detection range. Acetonitrile was the dilution agent in all sample dilutions. For soils, the first dilution was one gram of soil to 10 ml of acetonitrile yielding a 1 in 10 dilution factor; multiple extractions yielded correspondingly increased dilution factors. All aqueous dilutions were on a volume to volume basis including extracted TNT-solvent dilutions.

HPLC analyses were performed on samples diluted to a range falling within the linear portion of the HPLC

detector. Afterwards, the concentration determined for each sample was multiplied by its particular sample dilution factor to attain the actual sample TNT concentration.

Microbial Estimation

Numbers of microorganisms in soil and water samples taken from both field experiments were determined by Dr. James Entry and his staff in the AU Agronomy and Soils Microbiology Laboratory. To determine the number of bacteria in soil samples, a 1 gm soil sample was diluted in 9 ml of a phosphate buffer (pH 6.0) and shaken at approximately 120 rpm for 5 minutes. From this, a 1 ml aliquot was removed and stained for 3 minutes with 1 ml of a 20 $\mu\text{g ml}^{-1}$ FDA solution in a 0.2 M phosphate buffer. One ml of 1.5% agar in a pH 9.5 0.1 M phosphate buffer was added to the FDA suspension. The sample was mixed and an aliquot placed on a microscope well slide (Ingham and Klein 1984). Slides were examined for FDA-stained hypha length immediately after preparation by epifluorescent microscopy (Stamatiadis et al. 1990). Three fields per slide were examined with phase contrast microscopy for total hypha length, and three transects were examined for FDA-stained (active) hypha length at X1000 total magnification. Using epifluorescent oil-immersion microscopy, 5 fields per slide

were examined to determine numbers and size of fluorescent bacteria (Lodge and Ingham 1991). Fungi and bacteria are reported in numbers of microorganisms in one milliliter of water.

CHAPTER 4

FIELD EXPERIMENTS

Field Experiment Organization

Site selection

Originally known as the Alabama Ordnance Works, the Alabama Army Ammunition Plant (AAAP) was chosen as the experimental field site. It proved ideal for this research by possessing the necessary attributes of an available source of TNT contaminated soil, ready access to a water source, and obtainable indigenous aquatic plants in sufficient quantities for the study. Further, it met other important factors: the travel distance was not excessive, a significant volume of contaminated waste materials did not require transportation and storage off-site, and an experimental field site could be established near the source of raw materials. An overview of the industrial area showing the location of the field experiment site is included in the first chapter as Figure 1.2.

Site Layout

An area was cleared of small trees, bushes, and weeds using a small bulldozer. The clearing measured approximately 30 feet by 50 feet and was located about 100 feet from a beaver pond which provided the water source for the field experiments. A small greenhouse structure, 12 feet by 32 feet, was constructed on the cleared area.

One inch, schedule 40, PVC water pipe made up the greenhouse structural frame. Board runners made from pressure treated wood, one inch by four inches by 32 feet, were constructed and clamped to the pipe frame along both sides and down the middle. This provided structural stability by reducing the flexibility of the completed structure. Addition of a single layer of clear 6-mil plastic over the frame made the greenhouse complete. Structure orientation was roughly in the North-South direction along the longer axis. A photograph showing the completed structure is included in Appendix H as Photograph H1. This is an exterior view of the greenhouse and shows the ends covered for wind attenuation and heat retention during the winter months of the first field experiment.

Once the structure was completed, the soil solution containers were placed inside and flat on the ground. This ground contact more accurately simulated environmental field conditions. Soil solution containers were originally four

foot diameter children's wadding pools purchased from K-Mart and Wal-Mart.

Primarily, the purpose of the greenhouse structure was to provide a means for water level control in the soil solution containers. Also, its presence prevented the wasting of contaminated materials from the soil solution containers in heavy rainfall events.

Pond Water Characterization

The findings of an Inductively Coupled Argon Plasma (ICAP) analysis (Hue and Evans 1986) by the AU Agronomy and Soil Testing Laboratory of the pond water is listed in Table 4.1. No TNT was detected in the HPLC analysis of this pond water with detection limits below one ppm, approximately 0.1 ppm.

The pond has been in existence for many years and the water is clear with abundant aquatic life. Many aquatic organisms have been observed to include: minnows, insect larva, tadpoles and frogs, snails, snakes, water birds, and beavers. A photograph of the AAAP beaver pond that supplied water and Parrotfeather for both field experiments is included in Appendix H as Photograph H2.

Table 4.1

Pond Water Sample ICAP Analysis - Taken From the AAAP Beaver Pond Serving as the Field
Experiment Water Source

(Page 1 of 2)

	Ca	K	Mg	P	Cu	Fe
	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
	17.0	2.3	6.0	0.0	0.0	0.1
Detection Limit	0.01	0.3	0.02	0.06	0.02	0.06

	Mn	Zn	B	Mo	Al	Ba
	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
	0.0	0.0	0.0	0.0	0.0	0.0
Detection Limit	0.02	0.02	0.006	0.005	0.025	0.001

Table 4.1

Pond Water Sample ICAP Analysis - Taken From the AAP Beaver Pond Serving as the Field
Experiment Water Source

(Page 2 of 2)

	Co	Cr	Pb	Si	Na
	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
	0.0	0.0	0.0	3.1	2.5
Detection Limit	0.003	0.005	0.025	0.02	0.001

Plant Characterization

Commonly known as Parrotfeather, the plant selected for this research was the emersed aquatic weed, *Myriophyllum brasiliense*. It was found on-site at the AAAP and tentatively identified by Dr. Stephen Shimmel of Fountain, Florida during a preliminary survey. The plant had been previously identified by the ERL-A as having the nitroreductase enzyme and is listed in the Manual of the Vascular Flora of the Carolinas (Radford et al. 1968). Originally from South America, it was introduced into the United States as an aquarium plant and has escaped into waters throughout the southern part of this country (Tarver et al. 1978).

Parrotfeather

The Parrotfeather plants used in the field research were harvested from the same beaver pond on-site at the AAAP that served as the experimental water source. They were harvested from a water depth of two to three feet; most were gathered from a small area within 30 feet of the ponds south embankment. It is an aquatic plant that can be found in many local ponds throughout the year. Growth was abundant and the plants exhibited a healthy green color. However, lack of plant structural rigidity resulted in stem crimping

and bruising during the plant harvest, cleaning, and transport. Similar to a tomato bruise, these injuries caused the plant stem to soften at the injury and quickly undergo rot. Care was taken to minimize the impact of harvest and transport but the plants in both field and laboratory experiments extending over a eight week duration period often died.

Mostly, the Parrotfeather plant roots were attached to bottom sediments but many were also found affixed to tree debris, i.e., submerged and rotting tree sections. From a considerable root ball, the plant grows in a long strand or into branched strands. Looking down from the pond surface, the plants lay submerged just beneath the water surface and blanketed a large area of the pond. A photograph of a freshly harvested Parrotfeather strand is included in Appendix H as Photograph H3.

Plant Handling

A garden rake was used to extricate the plants rooted to the bottom sediments. Some stem breakage during harvest was unavoidable as the plant strands were intertwined. Dislodged plants were washed twice to remove tree limb debris, leaves, and sediments clinging to roots before being placed into the experimental containers. Various other foreign matter components were also removed including dead

plant segments; only healthy appearing plants or plant segments were selected and used.

The first wash was accomplished in the pond as the plants were harvested. This was followed by another clean pond-water wash in a pool located just outside the greenhouse. After washing, the plants were allowed to drip dry then weighed and distributed into the randomly preselected containers. Both field experiments had a plant to water ratio of approximately 1:60, weight (grams of plant) to volume (ml of water) basis. A photograph is included in Appendix H as H4 showing the second wash pool located just outside the greenhouse structure.

Contaminated Soil

Figure 1.5, shown in the first chapter, outlines the broad areas of potential contamination at the AAAP. However, several isolated spots of significant soil contamination are located within the areas depicted in this figure. These spots probably result from the land deposit of TNT and TNT containing materials that occurred during the time of active manufacturing operations, 1943 to 1945.

Soil Site

TNT contaminated soil used throughout this research was mined from a "bald spot", devoid of vegetation, located about 30 feet from a paved roadway in the "New TNT Area" at the AAAP. This is shown as area number 6 in Figure 1.5, Field Test Range and Contamination Map of the AAAP. Various species of vegetation, including pine trees and indigenous weeds, completely surround the TNT contaminated soil bald spot.

Contamination at this site is believed to result from past disposal practices where waste TNT was discarded; the site is located adjacent to the location where a "wash out" facility was once operated (Wynn 1994). Roughly circular, the bald spot has a diameter of about 30 to 40 feet. According to Mr. Wynn, AAAP site manager, the area of contamination has been reduced by half over the past twenty years with the encroachment of vegetation occurring from the outermost edge of the bald spot directed inward towards the center. Photographs of the contaminated soil site are included in Appendix H as H5 and H6. Photograph H5 is a view showing the extent of the site while Photograph H6 shows a closeup view of the soil surface with TNT nuggets clearly visible.

Analysis of soil taken from the selected site shows the TNT concentration in the soil to be about 4,000 parts per

million (ppm). However, small nuggets of TNT, often measuring 2 centimeters across the longest axis, are readily observable on the surface of the ground at this site. These nuggets exhibit a rusty red color due to environmental exposure; when broken apart, the unexposed substance inside the nugget appears as a yellow crystalline material.

Soil Preparation

TNT contaminated soil was excavated from the central portion of the bald spot for experimental use in this study. Only the top two to three inches were excavated as the higher TNT concentrations are believed to be in the upper portion of the soil profile. A high TNT soil concentration was desired in order to test the more extreme field condition. One of the chief parameters to observe was the effect of an actual contaminated soil on the natural water and plant system.

Once excavated, the soil was screened through a one-quarter inch mesh wire screen to remove twigs, leaves, stones, and clumps that could interfere with the sampling process. As it was screened, the prepared soil was stockpiled in one large pile; approximately 200 gallons of soil was screened, stockpiled, and covered by a sheet of 6-mil plastic for each field experiment.

Soil mixing resulted from the process of excavation, screening, and stockpiling the soil, however, a more thorough mixing was furnished by moving the entire stockpile by shovel, one shovelful at a time. Each stockpile of soil was mixed in this manner twice. After the second stockpile move, the entire soil stockpile was spread out on the plastic sheet and further mixed with a garden rake. Finally, the soil was re-shoveled into a pile. This process was used to thoroughly mix the soil for both field experiments. A photograph of the prepared TNT contaminated soil for the second field experiment is included in Appendix H as H7. The soil was stockpiled at the contamination site and this particular photograph was taken after soil mixing and before soil transport to the soil containers in the greenhouse.

Soil Field Handling

After the soil was thoroughly mixed, it was removed from the stockpile by shovel and placed into a wheelbarrow for transport approximately 1,500 feet to the greenhouse. There, the soil from each wheelbarrow load was removed by shovel, weighted, and distributed randomly to the prepared soil solution containers. A photograph is included in Appendix H as H8 showing an interior view of the greenhouse

as the soil is being measured and placed into the soil containers.

Each container received the same soil weights. Once each soil solution container had received its complement of soil, it was spread into a layer of approximately equal depth over the bottom of the container by a garden rake.

Soil Characterization

Representative contaminated soil samples for characterization analysis were taken after the soil was excavated, screened, stockpiled, and thoroughly mixed. A representative grab sample was randomly removed from each wheelbarrow load; these grab samples were removed just before the wheelbarrow contents were weighted and distributed into the soil solution containers. Enough soil was obtained from each grab sample to half fill a (3 X 2.5 X 4 inch) cardboard soil sample box. Two of these grab samples were then combined into a single sample for subsequent physical and mineral analyses.

Results from the soil analyses are disclosed in the following tables of both physical/chemical and mineral characteristics. Physical and chemical characteristics of the TNT-contaminated soil are listed in Table 4.2 and the contaminated soil mineral characteristics are listed in Table 4.3. Consistent with other soils in the area, the textural class is designated as a loam; it contains 38%

Table 4.2
 TNT Contaminated Soil Physical and Chemical Characteristics
 (Page 1 of 2)

Grab Sample Number	Particle Size (Percentage)			Texture Class	Water (3) Available
	Sand	Silt	Clay		
1	37.5	37.5	25.0	Loam	0.15
2	41.3	33.8	25.0	Loam	0.14
3	38.8	38.8	22.5	Loam	0.15
4	37.5	37.5	25.0	Loam	0.15
5	36.3	41.3	22.5	Loam	0.15
Average	38.3	37.8	24.0	Loam	0.15

Table 4.2
 TNT Contaminated Soil Physical and Chemical Characteristics
 (Page 2 of 2)

Grab Sample Number	Organic Matter (%)	Total		pH	Buffer pH
		N (%)	C (%)		
1	1.7	0.2	0.8	4.9	7.6
2	2.0	0.2	0.9	4.8	7.6
3	1.6	0.3	1.0	4.9	7.6
4	1.7	0.1	0.8	4.8	7.6
5	1.7	0.2	0.8	4.8	7.6
Average	1.7	0.2	0.9	4.9	7.6

Table 4.3

Results of Metals Analysis of TNT Contaminated Soil From the AAAP Mineral Characteristics

(Page 1 of 3)

Mineral Analysis (all values are ppm in soil)							
Grab Sample	Ca	K	Mg	P	Cu	Fe	Mn
1	590.5	58.2	104.1	6.1	1.7	16.9	23.2
2	580.4	57.4	102.1	5.8	1.7	16.1	23.3
3	558.9	59.5	101.9	5.9	1.7	19.5	23.7
4	570.6	59.8	103.2	5.6	1.8	19.1	23.8
5	602.0	60.2	107.1	6.0	1.7	19.0	24.3
Average	580.5	59.0	103.7	5.9	1.7	18.1	23.7

Table 4.3

Results of Metals Analysis of TNT Contaminated Soil From the AAP Mineral Characteristics

(Page 2 of 3)

Mineral Analysis (all values are ppm in soil)							
Grab Sample	Zn	B	Mo	Al	Ba	Co	Cr
1	3.2	0.3	0.1	58.9	6.6	0.2	0.2
2	2.2	0.3	0.1	52.0	6.5	0.3	0.2
3	2.5	0.3	0.1	59.2	6.2	0.3	0.2
4	2.5	0.3	0.1	61.7	6.6	0.3	0.2
5	2.2	0.3	0.1	61.1	7.2	0.3	0.2
Average	2.5	0.3	0.1	58.6	6.6	0.3	0.2

Table 4.3

Results of Metals Analysis of TNT Contaminated Soil From the AAP Mineral Characteristics

(Page 3 of 3)

Mineral Analysis (all values are ppm in soil)			
Grab Sample	Pb	Si	Na
1	37.8	89.1	25.1
2	38.5	79.9	27.3
3	37.9	81.3	26.5
4	38.1	95.9	24.8
5	39.7	104.0	25.6
Average	38.4	90.0	25.9

sand, 38% silt, and 24% clay. Also, the experimental soil was found to contain on average 1.7% organic matter by the Walkley-Black method (Walkley 1947) and a 4.85 soil-water pH. These methods are detailed in the "Reference Soil Test Methods for the Southern Region of the United States".

Field Sampling Methods

Aqueous and solid phase field samples were taken from the soil solution containers on a predetermined sampling schedule for the analysis of TNT concentrations. These samples were placed in a cooler for storage and transport from the field to the laboratory. All samples were refrigerated at the laboratory until prepared for analysis, usually within three days. A Kimble disposable 10 ml plastic pipette with a breakaway tip was the sampling device used to take representative samples from both the solid and aqueous phase.

Aqueous Phase

Aqueous samples were taken from each container with a 10 ml plastic pipette with a breakaway tip. The tip was detached and the cotton plug in the pipette top was removed to minimize liquid column disruption during sampling. Then, the pipette was slowly inserted through the liquid from the

top of the container water to just above the top of the soil surface. A finger was placed over the pipette top to seal the column so the sample would remain inside the pipette until it was transferred from the pipette to a 10 ml glass vial with a screw top.

This sampling process yielded a vertical water column sample with representative layers from the water surface to near the soil surface. Multiple samples taken from a single container showed the aqueous TNT concentrations were approximately the same magnitude throughout the container. Therefore, only one sample of about 5 - 8 ml was taken from each container for each sampling period.

Solid Phase

Soil samples were also taken from the soil solution containers via a 10 ml plastic pipette with a breakaway tip. With an inside diameter of 5/16 inch, the pipette was capable of encompassing the largest screened nugget that might be encountered in the experimental TNT contaminated soil. Care was exercised to insert the pipette vertically through the water and soil contents to the container bottom. Pressure applied to the pipette by a squeeze bulb was necessary to hold the sample inside the pipette while it was being removed from the soil stratum and the overlaying water layer. For the most part, the resulting sample provided a

representative specimen of the soil throughout the depth of the soil thickness. Each extracted soil sample was placed in a 3½ inch plastic culture cup having a loose fitting lid.

Field Soil TNT Extraction

Because the soil layers in the field experiments were covered by water, the entire depth of the soil medium was supersaturated. Therefore, all field soil samples were dried in a forage dryer at 60 °C for 24 to 48 hours to reduce solid phase sampling errors introduced by excess moisture.

After drying, a one gram dry soil subsample was removed from each sample and crushed to a powder. This one gram subsample was found representative of the complete sample as reported in the laboratory experiment chapter under the representative TNT-containing field soil subsample section.

Once dried and after subsample selection, the solid TNT material had to be dissolved to separate it from the soil matrix components. Jenkins and Grant (1987) report that acetonitrile is generally a good choice for a TNT solvent, so an acetonitrile TNT-extraction step was used to physically reposition the TNT from the solid phase into the

solution phase. This required that enough acetonitrile be added to each dry soil sample to extract the solid TNT.

To accomplish the extraction, the prepared soil subsample was placed in a capped screw top glass tube with 10 ml acetonitrile. Each soil and solvent containing tube was then secured in a test tube rack, mounted in a horizontal position to a beaker shaker. All samples were shaken at three revolutions per second for 24 hours, then allowed to settle for two to four hours. A 1.5 ml aliquot from each sample was removed by pipette and placed in a 1.5 ml plastic centrifuge vial and centrifuged on the Eppendorf 5415 for 10 minutes at 14,000 rpm to settle suspensions. A clarified 1 ml aliquot from each centrifuge vial was then filtered through a Whatman 0.2 μ m nylon syringe filter yielding a TNT extracted sample for subsequent dilution and TNT concentration analysis.

Field Experiment One

Field experiment one was designed to determine the ability of the plant Parrotfeather to phyto-remediate a TNT contaminated soil in a natural environment setting. This field experiment was accomplished with conditions as close to natural as possible and on-site at the AAAP in Childersburg, Alabama.

Experimental Method

Contaminated soil was placed in the soil solution containers located inside the greenhouse structure and pond water was added. There were a total of eight, four foot diameter, soil solution containers established with the contaminated soil and pond water.

Each container enclosed a 1½ to 2 inch layer of prepared TNT-contaminated soil; the layer equated to about 16 gallons measured volumetrically or 140 pounds measured as equivalent dry weight. Then, each container soil layer was topped by 6 to 8 inches of water; a total of approximately 50 to 60 gallons. The water was pumped unfiltered from the local beaver pond water directly into the containers.

After water addition followed by a one hour settling period, six of the containers were randomly selected for the addition of 10 pounds of the aquatic plant Parrotfeather. This equates to approximately a 1:60 plant to water (gm:ml) ratio in the containers with plants. Accordingly, the first experiment had two treatments, one treatment contained contaminated soil and pond water with Parrotfeather plants while the other treatment, considered a control treatment, contained contaminated soil and pond water without the Parrotfeather plants. This resulted in six repetitions for the treatment with plants and two repetitions for the control treatment.

Experimental Data Analysis

Soil and water samples were taken for routine TNT concentration analysis from 26 November 1993 to 28 February 1994. These were taken on a predetermined schedule; usually weekly.

TNT Concentration in Treatment Soils

Four soil samples were taken from each soil solution container at each prescheduled sampling time. One sample was randomly selected within each quadrant of each container and the corresponding TNT concentration determinations are listed in Appendix B, Table B1. This data table is sorted by pool container, sample quadrant, and sampling week with the "without plants" treatment data lightly shaded for emphasis.

To explore the relation between the resulting TNT concentration in the treatment soils for both "with Parrotfeather" and "without Parrotfeather" treatments, the entire data set was segregated first by sampling week, next by treatment, and finally averaged. This resulted in a single averaged treatment data point for the TNT concentration for each treatment and at each sampling time. This averaged soil TNT concentration data is presented in Table 4.4 with the corresponding plot of the data included as Figure 4.1.

Table 4.4

Field Experiment One: Averaged Soil TNT Concentration Sorted
by Time and Treatment Method

	Treatment Method	
Time	With Plants	Without Plants
(Weeks)	TNT Concentration	TNT Concentration
	(ppm)	(ppm)
1	10075	6442
2	4728	5645
3	4458	5347
4	4530	4602
5	3873	4926
6	4505	7748
8	5297	5360
9	4006	3772
10	4304	3976
11	4348	2950
13	4874	4601

Note: The "without plants" treatment data is lightly shaded
for emphasis.

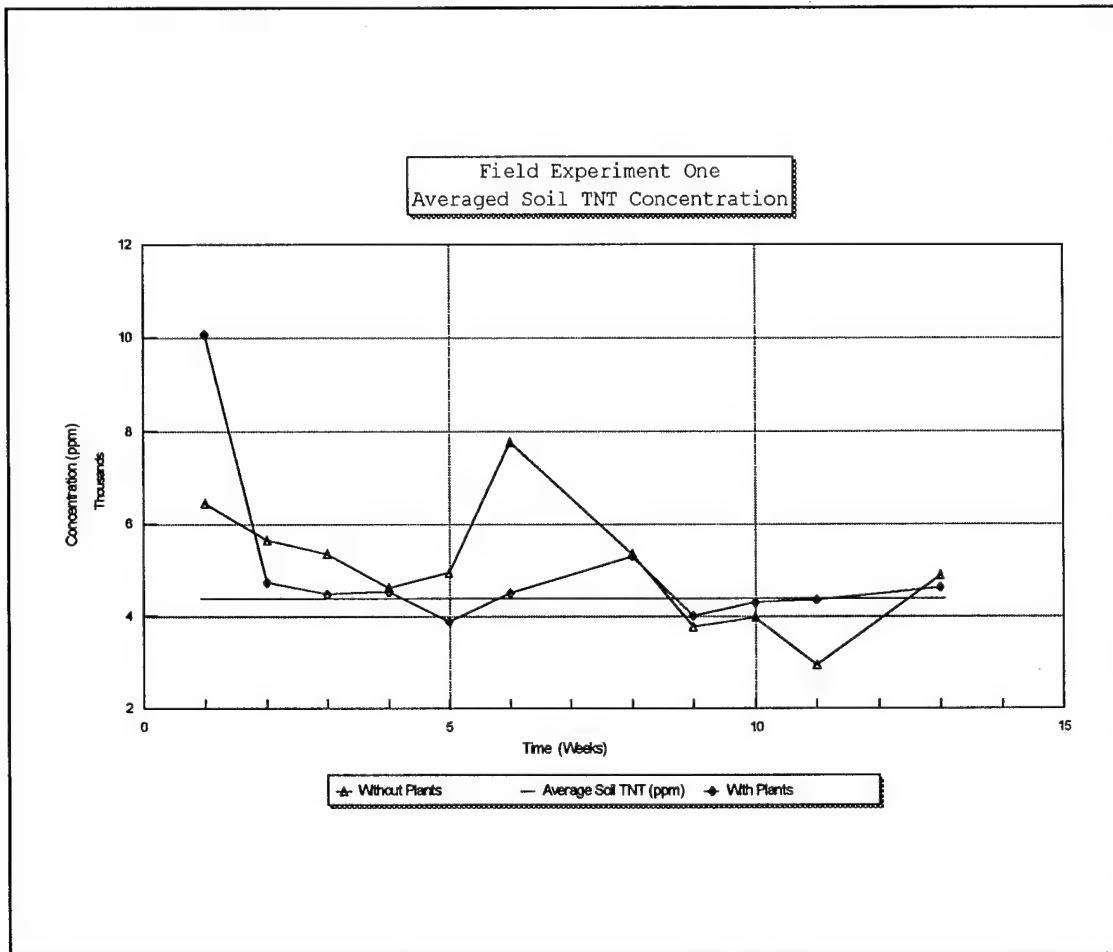


Figure 4.1

Average Soil TNT Concentration By Treatment

Visually, the data appears to show a slight trend of TNT concentration reduction in the soil for both treatments, however, the plot flattens out if the extreme data points are removed. Most likely, the absence or presence of TNT nuggets in the heterogeneous soil subsample caused the extreme concentration swings observed in the samples.

Actually, the averaged concentration values do not differentiate greatly from the average TNT concentration indicated by the horizontal line in the plot. This TNT concentration average was concluded from the laboratory analysis of a representative sample of the soil taken from the prepared soil stockpile used in this experiment.

Further evidence that the soil TNT contaminant concentration does not appreciably change comes from the lack of a statistically significant difference in the mean values of the two treatments. This determination was achieved by statistical analysis using the General Linear Methods model of the SAS Version Eight computer program at a 95% significance level.

During the analysis of this data it became obvious that the field sampling method employed for soil TNT concentrations was not effective for the limited duration of the field experiment. At an average TNT concentration of 4,376 ppm and equivalent dry weight of 140 pounds, there is roughly 250 grams of TNT in the soil within each

experimental container. With around 60 gallons of water added to each container, the TNT-water ratio was roughly 1000:1 in mg of TNT to liters of water. At this ratio, each container had enough TNT to achieve a maximum aqueous TNT concentration ten times.

However, because of the low solubility of TNT, the batch mode process chosen for the experiment, and mass transfer limits there was little driving force for a significant amount of the TNT to diffuse from the soil into the water. Most TNT dissolution into the overlying water likely occurred among the topmost portion of the soil layer. Due to slow rates and limited experiment duration, very little of the TNT within the depth of the soil matrix diffused into the aqueous phase so samples taken over the depth of the soil layer did not provide a viable measurement of the TNT concentration reduction. This process of intermediate TNT soil concentration sampling is only appropriate for experiments of a significant duration covering many months or perhaps years or for experiments with homogeneous materials.

TNT Concentration in Treatment Waters

One water sample was taken from each container for each sample period and analyzed for TNT concentration. Resulting data shows that Parrotfeather addition does significantly

reduce the TNT concentrations in water. These aqueous TNT concentration data, sorted by container pool and sampling week, are listed in Appendix B, Table B2. A statistical analysis by one-way ANOVA of this aqueous phase data shows a highly significant difference in the TNT concentration means between the treatments at the 95% confidence level.

This data, sorted and averaged by treatment, is summarized in Table 4.5. It is immediately obvious from the plot of this data in Figure 4.2 that the non-plant treatment contains about three times the amount of TNT in water as the plant treatment. Three very important details are evidenced from this data.

- First, there is a notable TNT concentration difference in the aqueous phase between treatments. From this, we deduce that the Parrotfeather treatment does cause a reduction in the aqueous phase TNT concentration.
- Next, the plant system functions to degrade TNT from water even in the cool winter conditions experienced in central Alabama. This increases the applicability of the method for in situ treatment opportunities.
- Finally, the maximum TNT concentration obtained in the control treatment was only one fourth of the ultimate value expected in water at 20 °C. It is known that TNT solubility is a function of temperature and lower temperatures result in a corresponding decrease in TNT

Table 4.5

Field Experiment One: Average Aqueous Phase TNT
Concentration Sorted by Time and Treatment Method

	Treatment	
Time	With Plants	Without Plants
Week	TNT (ppm)	TNT (ppm)
1	6	29
2	7	27
3	7	23
4	5	22
5	8	29
6	9	26
8	7	32

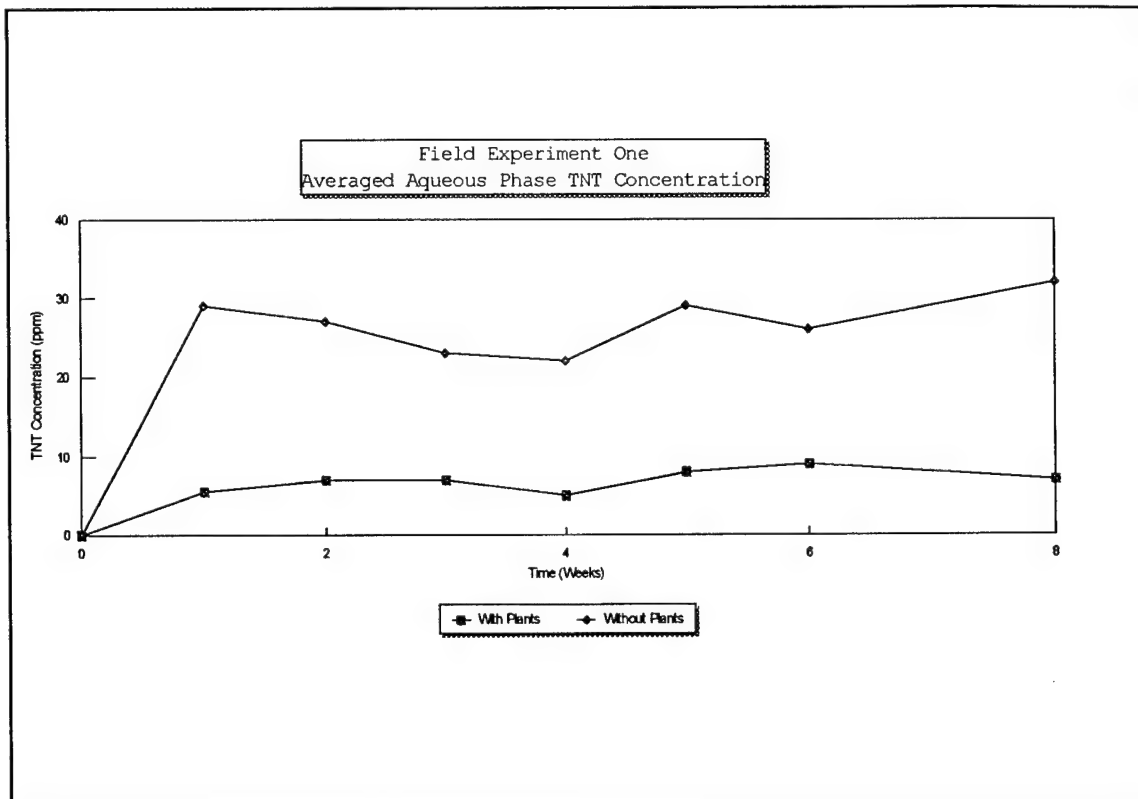


Figure 4.2

Field Experiment One: Average Aqueous Phase TNT
Concentration

concentrations in water. An investigation of the importance of temperature in the solubility of TNT is currently underway at AU (Bell 1994).

Field Water TNT-Product Identification

The presence of reduction products in the control treatment waters indicates some unidentified TNT reduction mechanism. Analysis of water from the "without plants" treatments by MECE (Ou 1994a), showed TNT, 2A and at least two other unidentified products. However, the "with plants" treatment analyses were found to contain TNT, 2A, 4A, 2,4DAT and several other unidentified products. Three possible means for the generation of the reduction products in the control treatments are nitroreductase enzymatic degradation, photolytic decomposition, and/or microbial degradation.

Number of Microorganisms in Soil and Water Field Samples

A microbial estimation was performed to determine if the bacteria and fungi populations in the field containers were sufficient to produce the observed products found in the water of the control treatment. Both soil and water samples were taken from each field container on week 20 for the analysis.

Results of the microbial estimate for both soil and water samples are sorted by container and listed in Appendix

B, Tables B3 and B4; the "without plants" treatment samples are lightly shaded. Regardless of treatment method, the data show that few active organisms were found in the soil samples of the field containers. Even though considerably more organisms were found in the water samples, they were at very low numbers. The average number of organisms for each treatment was found to be 221 and 150 for the "with plants" and "without plants" respectively.

This makes it extremely unlikely that microbial organisms were in sufficient quantities to metabolize a significant amount of TNT. As unfiltered pond water was the water recharge source for both treatments, the finding of active aqueous microorganisms was reasonable. However, the low numbers of organisms provide a direct indication that the treatment environments are toxic or inhibitory to these organisms.

Fungal activity was also checked but no fungi were found in either the soil or liquid samples. No tables of data are included due to fungi absence in these field samples.

Field Observations

A difference in the water color of the two treatments was the most pronounced observation. Coloration of the aqueous solution in all the containers with the

Parrotfeather added was clear but slightly turbid, whereas, the aqueous phase of the "without plants" treatment containers were conspicuously red. Still, both treatment containers were clear enough that the top of the soil layer was readily visible in all containers.

Also, a patchy but thick and scummy green mat developed in the containers with Parrotfeather toward the end of the experiment and was believed to be algae. No algae was observed in the non-plant treatment containers.

Problems Identified

An unknown mechanism apparently caused a reduction of TNT in the water of the control containers of the field experiment. This was evidenced by the presence of reduction products in the control water samples. Likely these resulted from microbial activities, pond water enzymatic reactions, and/or photolytic decompositions.

Another problem was the low ambient temperatures over the duration of the experiment and the effect this had on limiting the solubility of TNT. The highest and lowest temperature noted inside the greenhouse during this experiment was 80 and 22 °F, respectively. Also, the Parrotfeather likely become somewhat dormant in cooler waters although the results of this research shows TNT reductions continue to occur in the "with plants" treatment

even in the relatively low temperatures experienced in the Alabama winter.

Also, plant survival was limited; the plants began to darken in color with little evidence of new growth by week six after being placed in the soil solution containers. By week eight, all the plants were obviously dead and the containers were replanted. No attempt was made to place the aquatic plant roots into the soil layer as these aquatic plant roots are stated to be for anchorage instead of nourishment, unlike terrestrial plants. Plant survival difficulties could have been related to TNT or breakdown products but could also be due to plant harvest, handling, or transport shock.

The heterogenous nature of the soil was another significant problem; many TNT nuggets were small enough to pass the one quarter inch mesh sieve. When these nuggets were encountered in the soil samples they caused a huge deviation in the soil TNT concentration results. Additionally, if only a few nuggets are readily available for dissolution along the surface of the soil-water interface, soil sorbed TNT within the soil layer may not be solubilized. Nugget dissolution kinetics plays a significant role in heterogeneous TNT contaminated soil remediation.

Field Experiment Two

Field experiment two was also designed to determine the ability of the plant Parrotfeather to phyto-remediate a TNT contaminated media in a natural environment setting. However, the main focus of this field experiment shifted from the solid to the aqueous phase events as there was not enough time to observe an appreciable change in the two inch layer of TNT contaminated soil selected for the experiment. Additionally, enough information was gathered to formulate a series of laboratory experiments to confirm the Parrotfeather-TNT degradation process.

Experimental Method

All location and preparation conditions were the same as those in the first field experiment. However the number of soil solution containers was increased and the numbers of treatment repetition were balanced. This field experiment consisted of ten, four foot diameter, soil solution containers. Each contained 140 pounds as equivalent dry weight of soil. As in the first field experiment, this equates to approximately 2 inches of soil or about 16 gallons of soil in each container. Then, 72 ± 5 gallons of pond water was added to each container; roughly a nine inch depth of water. More water was added to the containers in

this experiment because of the greater expected evaporative water loss due to the warmer temperatures during this testing interval over the summer months.

After the water addition and a one hour settling period, five of the containers were randomly selected for the addition of 10 pounds of the aquatic plant Parrotfeather. Therefore, the second experiment had the same two treatments as the first experiment; one treatment contained plants and one did not. However, the treatment repetitions were balanced, five were with plants and five were without plants. Also similar to the first experiment, the plant to water ratio was roughly 1:60 on a gram to milliliter basis.

Experimental Data Analysis

Water samples were taken for routine TNT concentration analysis from 19 May 1994 to 6 September 1994. Also, soil and aqueous phase samples were taken from each container for a microbial numbers estimation during the second field experiment at week seven. Samples of fresh pond water and soil from the contaminated soil stockpile were included for baseline determinations. These samples were placed in a cooler and transported to the AU Agronomy Soils Microbiology Laboratory for analysis.

TNT Concentration in Treatment Waters

All aqueous phase TNT concentration data from this second field experiment, sorted by pool container and week, is tabulated in Appendix C, Table C1. As in the first field experiment, the aqueous phase TNT concentration data was sorted by treatment and averaged by week sampled. This averaged data is listed in Table 4.6 with the corresponding plot of the concentration over time shown as Figure 4.3. It is apparent that aqueous phase TNT was quickly removed from the containers with the Parrotfeather treatment, whereas, the non-plant containers continued to show increasing levels of TNT in the aqueous phase over the duration of the sampling period.

Most likely, the agitation resulting from the original water addition to the test containers already having the soil in place resulted in an initial dissolution of TNT into the water. Afterwards, the plants removed this initial solubilized TNT in those containers having the plant treatment and any additional TNT as fast as it diffused into the water. However, the non-plant treatment showed an increasing TNT concentration as the TNT continued to diffuse from the soil into the water. It is possible that the

Table 4.6

Field Experiment Two: Averaged Aqueous Phase TNT
Concentration Sorted by Time and Treatment Method

Field Experiment Two Averaged Aqueous Phase TNT Concentration		
Time Weeks	Treatments	
	With Plants	Without Plants
	(ppm)	(ppm)
0	0.0	0.0
1	0.4	7.8
2	0.3	7.4
3	0.1	9.4
4	0.4	10.0
5	ND	12.6
7	0.1	13.6
15	ND	18.0

Note: Values listed as ND = None Detected.

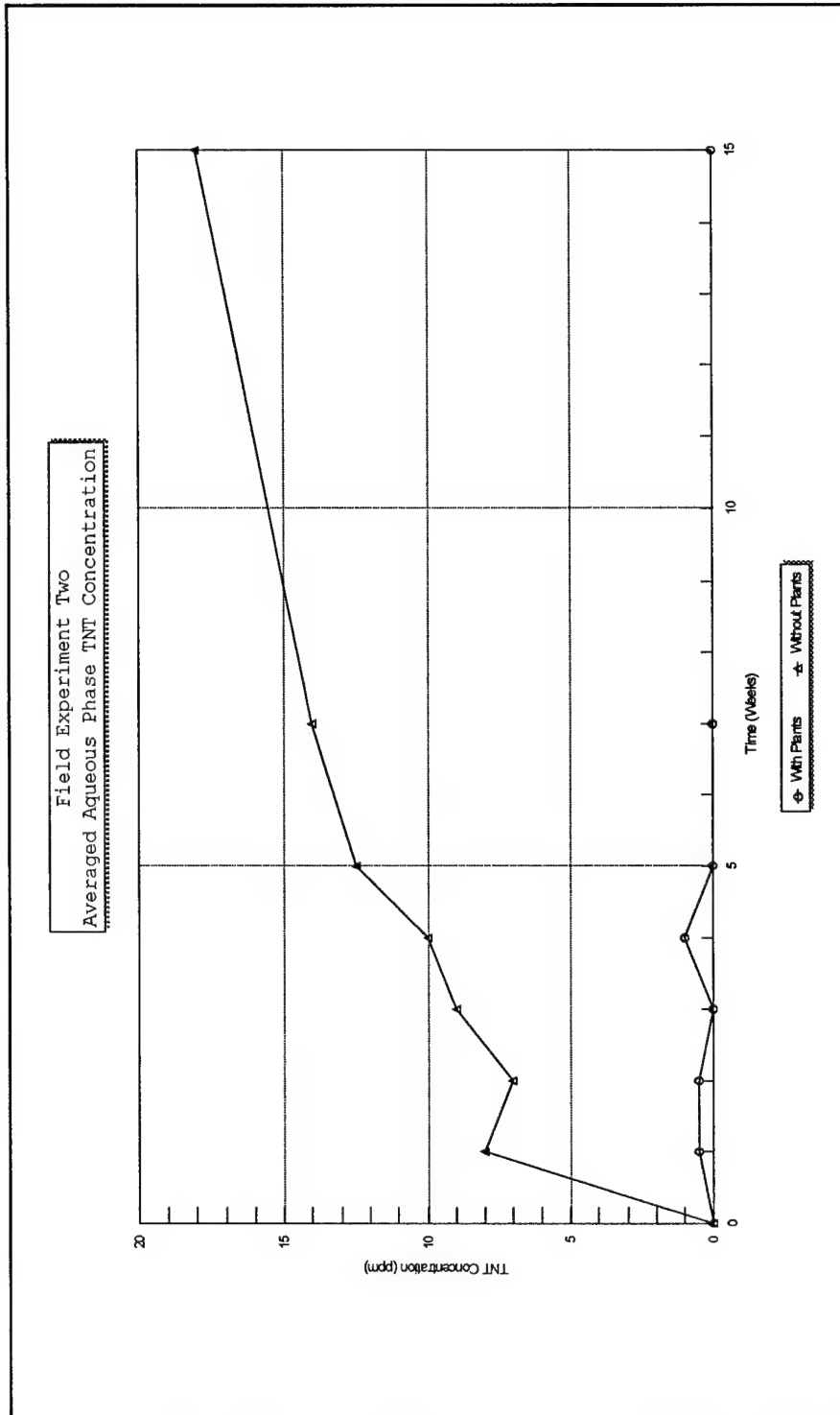


Figure 4.3

Field Experiment Two Averaged Aqueous Phase TNT Concentration

maximum TNT solubility would be reached in these containers given enough time or the application of sufficient agitation.

Both treatments, in this experiment, showed lower aqueous TNT concentrations than were found in the first field experiment. Most likely, this was due to the increased plant activity during the warmer months and reduced agitation from initial water addition. However, the increased photolytic and microbial opportunities could have also contributed to the aqueous TNT decrease.

TNT and Product Identification in Field Water

In addition to the TNT concentration determinations, a set of aqueous samples were taken over the entire course of the second field experiment from both treatments and analyzed for TNT and reduction product appearance and disappearance by MECE. These data are shown in Table 4.7 and Table 4.8 for the "with plants" and "without plants" treatments respectively.

These provide confirmation by MECE chromatogram that the Parrotfeather treatment in the field experiment results in the formation of monoamino (2A & 4A) and diamino (2,4 & 2,6) isomers. Although no TAT was specifically identified in this analysis, the reduction process is analogous to Stonewort (Wolfe et al. 1994) and most likely the reaction

Table 4.7

Field Experiment Two: MECE Analysis of the TNT and Reduction Product Concentrations in the Aqueous Phase of the "With Parrotfeather" Treatment

Field Experiment Two: "With Parrotfeather" Treatment						
Time (Week)	TNT and Reduction Compound(s) Concentration (ppm)					
	TAT	2,6DA	2,4DA	TNT	2A	4A
1	ND	0.4	2.1	0.3	3.6	5.3
2	ND	1.0	2.1	0.0	0.9	0.8
3	ND	0.5	0.3	0.0	1.4	1.0
4	ND	0.2	0.3	ND	1.9	1.0
5	ND	0.6	0.6	ND	1.9	0.8
7	ND	0.4	0.5	ND	4.5	2.7
15	ND	ND	ND	ND	ND	ND

Table 4.8

Field Experiment Two: MECE Analysis of the TNT and Reduction Product Concentrations in the Aqueous Phase of the "Without Parrotfeather" Treatment

Field Experiment Two: "Without Parrotfeather" Treatment						
Time (Week)	TNT and Reduction Compound(s) Concentration (ppm)					
	TAT	2,6DA	2,4DA	TNT	2A	4A
1	ND	ND	ND	11.6	4.8	ND
2	ND	ND	ND	6.9	7.2	ND
3	ND	ND	ND	8.0	6.0	ND
4	ND	ND	ND	11.0	7.4	ND
5	ND	ND	ND	9.4	8.8	ND
7	ND	ND	ND	21.0	11.4	ND
15	ND	ND	ND	19.0	7.8	ND

pathway of aqueous TNT by Parrotfeather precedes through the nitro group reduction to the TAT with subsequent oxidation ultimately yielding mineralization products. One example chromatogram from each treatment was selected from this analysis and is presented as Figure 4.4 and Figure 4.5 for the "with plants" and "without plants" treatments respectively.

Also, from this analysis confirmation was obtained for 2A as the major and single amino product found in the non-plant control treatments. Because the major identified photolytic products (Burlingson et al. 1979) do not include amino compounds, and because the microbial counts are so low, the control treatments reduction products most likely result from nitroreductase enzyme actions from the pond water.

UV Spectral Profiles

A UV spectral scan of the products from the aqueous phase of both treatments in this field experiment was accomplished after separation of the components by MECE and obtaining the UV-VIS spectra using a diode array detector (Ou 1994b). Spectral profiles of products resulting from TNT and reduction product standards are shown in Appendix E, Figure E1 through E6. Each profile is annotated to indicate the peak retention time and the product. Actual sample

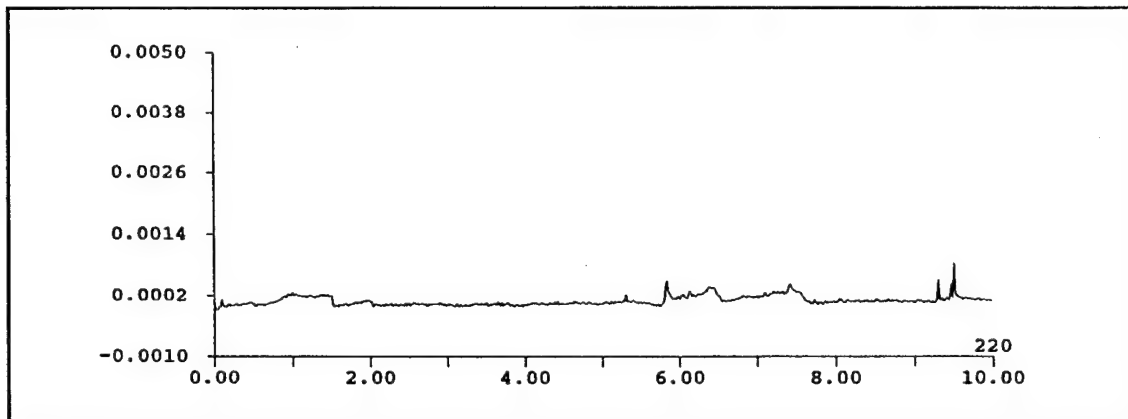


Figure 4.4

MECE Chromatogram of a Field Experiment Two Water Sample
Taken at Week 15 From the "With Plants" Treatment.

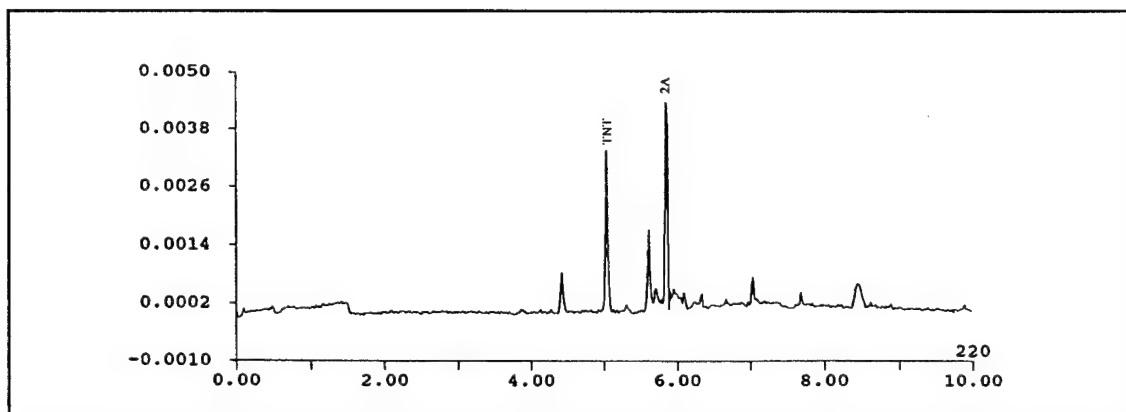


Figure 4.5

MECE Chromatogram of a Field Experiment Two Water Sample
Taken at Week 15 From the "Without Plants" Treatment.

spectra are not included as they match those shown for the standards. These spectra derived from this field experiment do provide another verification that the "with plants" treatment shows the presence of monoamino (2A & 4A) and diamino (2,4 & 2,6) isomers while the "without plants" treatment shows only TNT and the monoamino products.

GC/MS Product Identification

To insure that the retention time and spectra identification of TNT and the reduction products were correct, one water sample from each field treatment was taken for concentration and subsequent analysis by GC/MS (Cipollone 1994). A Waters Sep-Pak C-18 cartridge was used to extract and concentrate the TNT and products from the original five ml water sample taken from the second field experiment on the fifth week.

The resulting chromatogram, annotated to identify reduction products, from the "with plants" treatment is shown as Figure 4.6. Both monoamino and diamino isomers were clearly evident but TNT was conspicuously absent. TAT was not specifically identified in this sample but under oxidative conditions it does not build up and is extremely transitory so its absence was not unexpected.

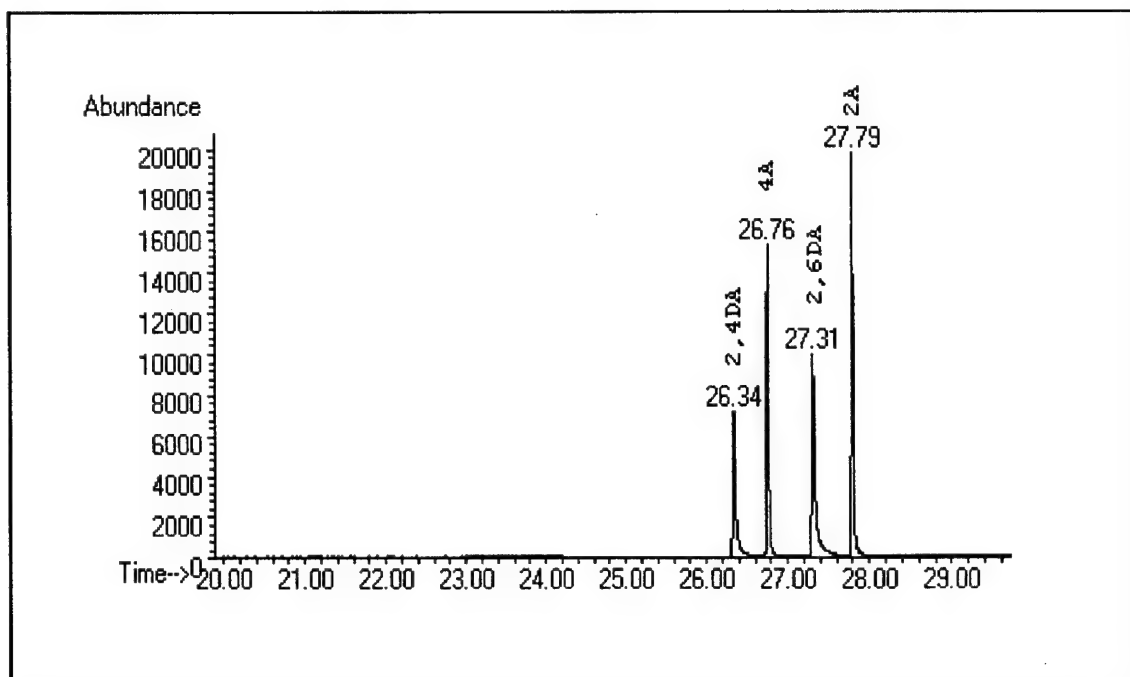


Figure 4.6

GC/MS Chromatogram of Field Experiment Two Aqueous Phase
Parrotfeather Treatment Sample With Identified Reduction
Product Peaks Annotated

The other chromatogram resulting from the "without Plants" treatment is also annotated and presented in Figure 4.7. Only the TNT and monoamino isomers were found in this sample. These monoamino TNT breakdown products probably result from enzymatic activity present in the treatment pond water recharge source as the microbial counts are low and the pond water was found to contain nitroreductase activity (Carreira 1994).

Number of Microorganisms in Soil and Water Field Samples

To better determine the microbial contribution to TNT degradation, both water and soil samples were taken from each container at week seven for an estimation of the total number of microorganisms. These data for soil and water samples are included in Appendix C, Tables C4 and C5. Analogous to the first field experiment, these data also show no observable active fungi in either soil or liquid phase samples. Also similar to the first field experiment, there were few observable bacteria in the soil samples taken from the field containers or from soil taken fresh from the contaminated site.

Analysis of the aqueous phase data from this field experiment, presented in Appendix C Table C5, reveals that the average number of organisms found in the water of the "with plants" treatment was 203, in the "without plants"

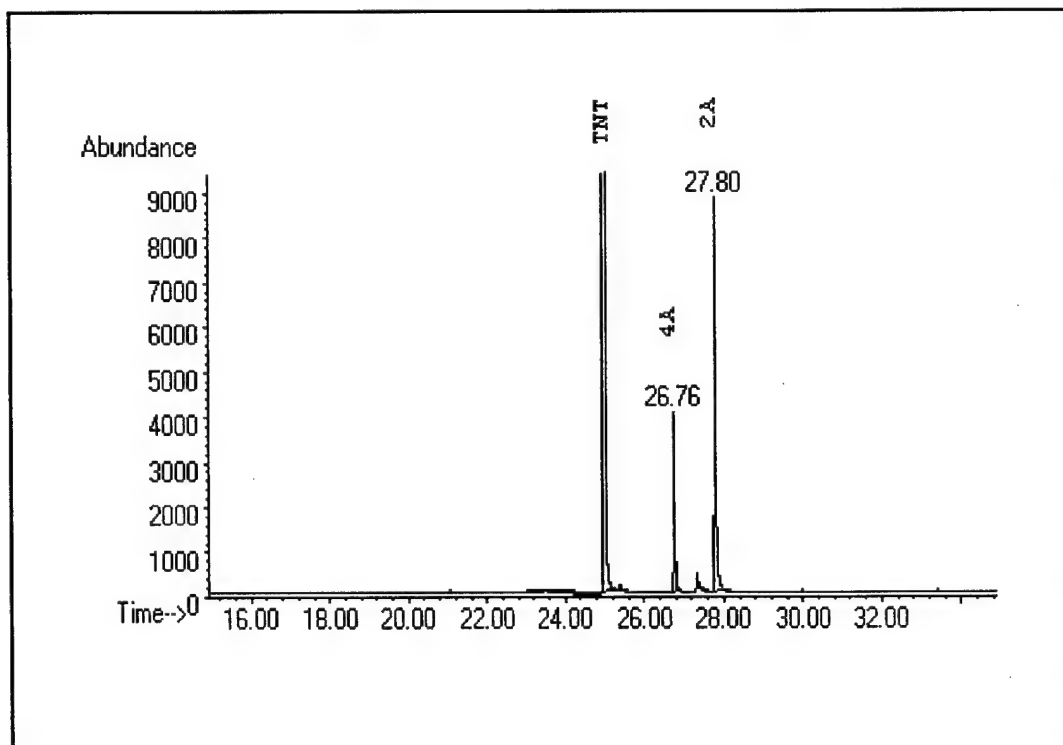


Figure 4.7

GC/MS Chromatogram of Field Experiment Two Aqueous Phase
Without Parrotfeather Treatment Sample With Identified
Reduction Product Peaks Annotated

treatment was 77 and in the raw pond water was 520. Thus, there was a marked decrease in the aqueous phase bacterial numbers in the "without plants" treatment; they were reduced by 85% when compared to the raw pond water numbers.

Although the bacterial numbers were greater in the water of the "with plants" treatment, there was a 62% reduction in the numbers of bacteria when compared to those from the raw pond water.

Fresh pond water was the water recharge source for both treatments with about ten gallons routinely added per week so TNT and/or an unknown breakdown product(s) is likely responsible for the decline in bacterial numbers. Regardless of the agent, something in the water is toxic or inhibitory to these organisms resulting in their decreased numbers. Significantly, the "with plants" treatment apparently lessens the effect.

Nitroreductase Estimation

Samples of both water and soil from each treatment and container in the second field experiment were prepared and analyzed for nitroreductase activity by the methods developed at the ERL-A (Carreira and Wolfe 1994). Although no clear pattern was evident regarding the nitroreductase estimation in these samples, there was nitroreductase activity found in most samples; little was evidenced in the

soil samples and the higher amounts in water were in the without plants treatments. This data is shown in Table 4.9.

Field Observations

Aqueous phase coloration differences were even more pronounced in this experiment as the color of the water in each non-plant containers was a much deeper color. While the water in the containers with Parrotfeather remained colorless and slightly turbid, the non-plant water turned a deep burgundy color. This burgundy color was dark enough to be opaque; the top of the soil layer was not visible through the water. TNT colored complexed compounds generated by photolytic transformations are known to result in similar colors (Burlingson et al. 1979). As these colors are not generated in the treatment waters containing the Parrotfeather plants, the plants have the ability to inhibit or eliminate the compounds causing the color. Photographs are included in Appendix H as H9 and H10 that show the experimental containers at two different times during the experiment. Photograph H9 shows the experiment just after the addition of Parrotfeather plants. Photograph H10 shows the same view taken one week later.

Another significant observation was that active aquatic organisms were frequently observed in the water of the field containers containing plants. They were first observed at

Table 4.9

Field Experiment Two: Nitroreductase Enzyme Estimate

Parrotfeather Nitrate Reductase Estimate		
1.4 mg/ml total protein (1:1 extraction)		
	Nitroreductase	
	(ng/ml)	(ng/ml)
Container	Soil	Water
1	None Detected	None Detected
2	None Detected	10
3	16.5	10
4	10	10
5	10	None Detected
6	None Detected	8
7	None Detected	10
8	4	None Detected
9	None Detected	4
10	None Detected	12
Pond Water		12.5

Note: Shaded data indicates "without plants" treatment.

week five; in the latter part of June. Observed organisms include tadpoles, aquatic snails, and various insect larvae. Two specie of tadpole were evident. Many were small and black, however, the majority were a bigger variety having a muddy brown color with small spots down the tail. No aquatic organisms were ever observed in any of the non-plant containers.

Similar to the first field experiment, the plants perished. By week five after being placed in the soil solution containers, plants in two of the five "with plant" treatments began to darken in color and exhibited no visible new growth. By week seven, these plants were dead and the plants in other three containers were obviously distressed. Between weeks seven and ten no samples were taken and by week ten the plants had all expired; no green shoots were visible and the clear liquid in the containers that previously contained live plants had turned a light red color. All plant treatment containers were replanted with another ten pounds of Parrotfeather at week ten.

Another pool was located outside the greenhouse and used for the second plant wash just prior to the drip dry step. Excess plants harvested from the pond at the beginning of the experiment were placed directly from the pond wash into this outside pool. These plants never underwent the additional wash and drip dry process or TNT

exposure. They appeared healthy at the ten week point even though all the treatment plants were dead. Considering the outside pool as a disturbed control, this indicates that either the additional wash and drip dry process or some component of the TNT or TNT products contributed to the plants demise.

As observed in the first field experiment and toward the end of this experiment, the "with plant" treatment waters developed a scattered scummy and green mat while the non-plant waters did not. As this is most likely composed of algae, it indicates that something in the treatment without plants is inhibitory or harmful to algae.

Problems Identified

Field TNT transformations were found to result from avenues other than exposure to the plants. Major means of degradation include, Parrotfeather plants, sunlight, and pond water. Parrotfeather initiated TNT reductions were likely a result of the plant produced nitroreductase enzyme activity and became the parameter of primary interest. However, the pond water was assayed for nitroreductase enzyme activity and found to contain half as much of the plant nitroreductase enzyme as was found in the plant (Carreira 1994).

Although the photolytic transformation of TNT may not result in a massive reduction in the TNT concentrations in water, it must be thoroughly investigated to determine the quantity, composition, and toxicity of the colored complexes. This information will prove important in any in situ remediation scheme involving aquatic plants because of the plant photolytic requirements.

The highest TNT concentration obtained was only about one fourth of the 20 °C expected value. During the first five weeks, the average high and low temperatures recorded inside the greenhouse were 110 and 60 °F respectively. As the ambient temperatures were significantly higher during this sampling period, the aqueous phase TNT concentration levels were also expected to be much higher, however, this was not the case. Likely the unexpectedly low TNT concentrations resulted from the slowness of the diffusion of TNT from soil into water rather than a solubility limitation.

Because the aqueous TNT concentration evidenced a slow but continuous increase in the control treatments under the batch mode process, mass diffusion of TNT from the soil matrix into the water is likely the rate limiting step in this process. Thus, because Parrotfeather quickly removes TNT from water, the key to greatly improve the efficiency of

this method is to increase the movement of the TNT into the water.

Field Water pH Determinations

Aqueous phase pH measurements were taken in the field during the second field experiment. These data, after sorting by week sampled and pool container, are shown in Appendix C, Table C3. Because the measurement variations within the individual containers were insignificant, only one reading was taken from each container. Also, weekly readings were not taken as the pH did not vary significantly within each treatment or change appreciably over time.

This data in Table C3 is averaged by treatment and presented in Table 4.10 with the corresponding low and high pH values indicated. Comparisons between low, average, and high values show that little variation was found in the pH measurements within the individual treatments. A plot of the averaged data is shown in Figure 4.8. The "without plants" treatment remained at a roughly neutral pH while the other "with plants" treatment was consistently around a half pH unit lower.

As the TNT contaminated soil pH was 4.8, the pond water likely provided the buffering that elevated the water pH in the "without plants" treatment. From the literature reviewed, the impact of pH, whether negative or positive,

Table 4.10

Field Experiment Two: Averaged pH Values Sorted by Week and Treatment

Field Experiment Two - pH Values						
	With Plants Treatment			Without Plants Treatment		
Week	Low	Average	High	Low	Average	High
1	6.5	6.5	6.6	7.0	7.1	7.1
2	6.5	6.6	6.8	7.0	7.0	7.0
4	6.6	6.7	6.8	7.1	7.1	7.2

Note: pH data values in the "without plants" treatment is lightly shaded for emphasis.

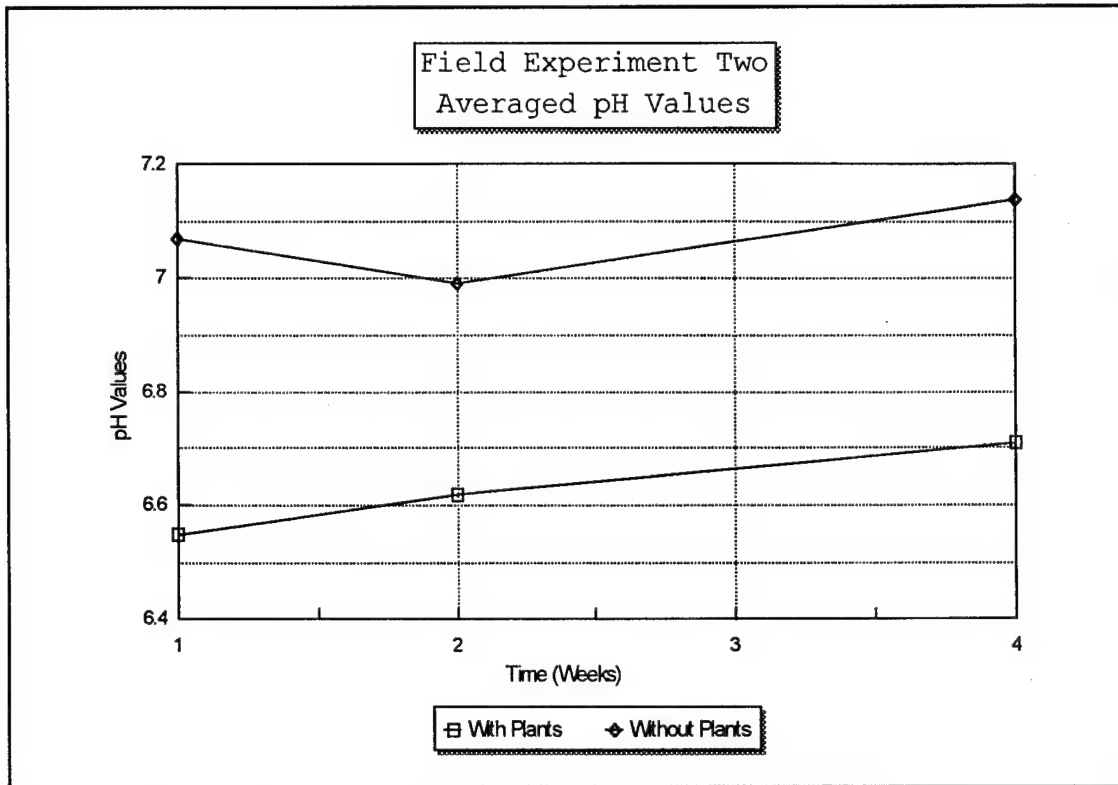


Figure 4.8

Field Experiment Two Averaged pH Values

lies mainly with its importance to the microbial population and photolytic transformation process.

Soil Redox Potentials

Probes were constructed, calibrated, and installed to determine the combined redox potential of the field container systems. Although measurements are typically adjusted for reporting at a neutral pH (Bohn 1971), these Eh measurements were not adjusted for pH because little variation from neutral occurred. However, redox potential readings are also normally based on the standard hydrogen reference electrode instead of the saturated calomel reference electrode. Accordingly, the field Eh readings were adjusted to the hydrogen standard; +244 mV was added to each of the meter readings taken with the saturated calomel reference electrode (Faulkner et al. 1989).

After the measurements were adjusted to conform to the standard hydrogen reference electrode, they were sorted by week taken, pool container, and location within the pool and presented in Appendix C, Table C2. Unfortunately, the measurements were terminated during week seven sampling due to a meter malfunction. A loose connection was determined to be the culprit and was soon repaired, however, other priorities prevented additional readings. Weeks four, five and seven average Eh values were: 301, 239, & 134 for the

"with plants" and 375, 309, & 278 for the "without plants" treatments.

Although insufficient data was gathered to state uncategorically and with any degree of certainty the exact state of the environment, the systems appeared to be slowly going anaerobic. An oxidized soil has redox potentials in the range +400 to +700 mV (Patrick and Mahapatra 1968) and the averaged values in the field containers were well below this range. Further, sediment and submerged soils usually have redox potentials from -400 to +700 mV, strongly reduced to highly oxidized respectively (Gambrell and Patrick 1978).

CHAPTER 5

LABORATORY EXPERIMENTS

Laboratory Materials

All laboratory materials are the same as those previously identified in the field experiments chapter. However, laboratory preparation of the soil and plant materials did differ from their preparation in the field experiments.

Laboratory TNT Contaminated Soil

TNT contaminated soil for each laboratory experiment originated from the stockpile of contaminated soil prepared in the first field experiment. A grab sample of roughly 500 grams was taken to the laboratory and prepared as the experimental soil. Preparation consisted of drying the soil in a forage dryer at 60 °C for five days and then crushing the entire quantity of soil to a fine powder by mortar and pedestal. Finally, the powdered soil was passed through a number 20 sieve, thoroughly mixed, and stored until needed for specific laboratory experiments. This soil preparation was necessary to facilitate solute and solvent contact and

to provide a homogeneous soil by elimination of the TNT nuggets.

This prepared soil was used in most laboratory experiments as a source of TNT contamination. To determine the TNT concentration contained in this prepared soil, five random samples were taken and assayed for TNT concentration using the multiple extraction technique. Analysis of the resulting data shows the soil to contain an average TNT concentration of 4,376 ppm with a standard deviation of 338 ppm.

Laboratory Plants

Parrotfeather plants were harvested fresh for each laboratory experiment; they were taken from the same beaver pond at the AAAP that provided the plant and water sources for both field experiments. They were pond-water washed and gently placed in five gallon buckets containing pond water for transport to the laboratory. Before use in laboratory experiments, the plants were gently washed in the laboratory with distilled water to remove any remaining detritus. They were washed at least twice and until the wash water was visually clear and debris free. All experimental plants were measured as a blotted dry weight.

Laboratory Sampling

Different methods were used for sample withdrawals in the laboratory than were used in the field as described below. These changes were necessary, for the most part, because of the significantly reduced volumes of materials involved.

Aqueous Phase

Generally, for aqueous phase laboratory sampling, one ml subsamples were removed by a pipette with a disposable plastic tip at predetermined times. They were transferred from the pipette to 1.5 ml plastic centrifuge vials and refrigerated if not prepared for immediate analysis.

Aqueous sample preparation prior to TNT concentration determination was centrifugation on the Eppendorf 5415 and subsequent dilution. After centrifugation at 14,000 rpms for 10 minutes, a clarified aliquot was removed by pipette and placed in another 1.5 ml plastic centrifuge vial for dilution with acetonitrile. Afterward, each sample was mixed for one minute by a vortex mixer on the highest setting. Approximately 350 μ l of this dilution was placed in the plastic autosampler vial and capped ready for HPLC analysis.

Solid Phase

The most significant change between the field and laboratory experiments occurred in the methods of soil sample collection and preparation. A critical drawback identified in the first field experiment was the intermediate sampling of contaminated soil over a very limited duration period and in the presence of an huge heterogeneous source (swamping) of pollutant.

Therefore, intermediate soil samples, as a function of time, were not taken from the contaminated soil in the laboratory experiments. Initial soil TNT concentrations were determined from random one gram samples taken from the previously prepared and stored laboratory soil. Once this data was averaged, it represented the initial soil TNT concentration and could be compared to the final averaged concentrations determined when the individual sample sets were terminated.

Also, the overall process for soil sample preparations remained essentially the same as those used in the field experiment except for the TNT extraction step. As the single extraction method used in the field experiment was not deemed able to adequately extract the TNT from the soil, a laboratory experiment was conducted to find a better method. Results of a Laboratory experiment, using the contaminated soil, showed a multiple extract procedure to be

able to more fully extract TNT from the soil. This TNT-soil extraction laboratory experiment is thoroughly detailed in Appendix A. Each laboratory experiment involving soil TNT concentration determinations used this multiple extraction method.

Similar to the field experiments, the experimental soil samples were saturated with water and required drying. Before drying, the laboratory soils were filtered through a Genuine American Grade 802 Filter Paper. Plant materials were removed from any samples containing plants before filtration. After filtration, the liquid was decanted off and the solids were allowed to air dry for 5 days. After the dry solids were re-crushed and thoroughly mixed, one gram subsamples were taken for sample TNT concentration determinations by the multiple extraction method.

After the first field experiment, the multiple extraction procedure was found to provide a more complete extraction of TNT from the contaminated soil. Although this procedure required more acetonitrile, it was quicker. After the soil preparation for extraction, the sample was placed in a screw top glass tube with 10 ml of acetonitrile solvent. Then, each tube was mounted in a test tube rack and placed in a vial shaker, in a horizontal position, and agitated at five revolutions per second for 30 minutes to enhance the TNT mass transfer into the solvent. After

agitation, the TNT-solvent solution was removed from the tube. This process of solvent addition, sample agitation, and solution removal was executed three times and shown to be adequate by the laboratory experiment detailed in Appendix A. Three TNT-solvent extractions of each sample was combined in a single test tube and mixed by vortex mixer for one minute. After a short settling period, a one ml aliquot of the liquid was removed and centrifuged in preparation for dilution and subsequent TNT concentration determinations.

Laboratory Experiments

Laboratory experiments were conducted under controlled conditions to better quantify, explain, and expand the field results. Most were conducted in the laboratory under a controlled temperature of 23 °C and with standard lighting. All TNT concentration determinations were made by reverse phase HPLC. Also, each experiment was specifically designed and performed to resolve pertinent questions that arose during the course of the field investigations. Questions such as, how fast does the plant degrade aqueous phase TNT? And field materials effect on the resulting microbial population? As well as, what is the resulting fate of TNT due to Parrotfeather mediated breakdown? The resulting data

from these experiments authenticates the ability of the Parrotfeather to generate TNT degradation.

TNT Containing Field Soil - Representative Subsample Determination

Because of the heterogenous nature of the TNT nugget-containing field soil, an early experiment was performed to determine the amount of soil subsample that best represented the soil population; one, five, or twenty grams. Experimental results are sorted by quantity of initial soil sample and listed in Table 5.1. Statistical analysis of this data, by a one-way analysis of variance (ANOVA) with a 95% confidence level, shows the amount of soil solids initially selected for the subsample, in this experiment, did not significantly impact the resulting average TNT concentration. Therefore, a one gram subsample of this contaminated soil rendered as valid a result as a 20 gram subsample. Therefore, each subsequent soil TNT extraction was performed on a one gram subsample.

Microbial Analysis in the Presence of TNT

A laboratory experiment was designed and performed to elucidate the degradation of TNT by soil and/or pond water microorganisms found in the field experiment treatments. A series of beakers containing the native contaminated soil

Table 5.1

TNT Contaminated Field Soil - Representative Subsample

Determination of TNT Concentration

Amount of Initial Soil Subsample		
1 gram	5 gram	20 gram
TNT (ppm)	TNT (ppm)	TNT (ppm)
4558	4251	4334
4689	7851	5931
4419	4548	5798
4744	4872	5065
4795	4334	6660
5171	5008	4790

from the selected site of TNT contamination from the AAAP was prepared. Both sterilized and non-sterilized soil were used to better assess the affects directly attributable to soil microorganisms. Likewise, a series of sterilized and non-sterilized pond and tap waters were incorporated into the experiment.

Methods and Materials

Sterile contaminated soil was generated by exposure to an atmosphere that was saturated with chloroform for 24 hours (Parkinson and Paul 1982). Also, both types of water were made sterile by placement in an autoclave at 20 psi and 120 °C for one hour. The pond water for this experiment was obtained from the same beaver pond as the Parrotfeather plants and used within two days after its removal from the pond. Tap water used in this experiment was taken directly from the AU laboratory tap.

The contaminated soil for this experiment originated from the contaminated soil stockpile used in the first field experiment. To better emulate the field experiment, the non-sterile TNT nugget-containing soil was not specially prepared for this experiment; it was taken directly from the stockpile, weighted, and added to the containers.

Experimental plants were quantified as a blotted dry weight and were naturally unsterile. The plants were pond

water washed during harvest and placed in five gallon buckets of pond water for transportation to the lab. Care was taken to select only healthy plants which were then gently rinsed with distilled water and quickly blotted dry.

All the environment beakers were 250 ml flasks with number six rubber stoppers. Beakers and stoppers were sterilized by autoclaving at 20 psi and 120 °C for twenty minutes prior to the addition of TNT contaminated soil and water. Each test beaker received 50 grams of contaminated soil, measured as equivalent dry weight, and 100 ml water. As the selected experimental parameters included two types of soil, three types of water, and selected treatments received plants, a matrix of the experimental environments included in this experiment are detailed in Table 5.2. A total of 24 flasks were established comprising eight treatments with three repetitions.

All work was performed aseptically under a laminar flow hood to prevent contamination. Plants were added to selected flasks at a plant to water ratio of 1:20; 5 grams of Parrotfeather to 100 ml water. After all the constituents were added, the beakers were capped with sterilized number 6 rubber stoppers having a single hole and the hole was filled with cotton to minimize evaporation losses. All beakers were then incubated inside a lighted hood for ten weeks.

Table 5.2

Microbial Analysis in the Presence of TNT - Matrix of Experimental Treatments Investigated

Treatment	Soil	Water	With Plants
1	Sterile	Sterile Pond	No
2	Sterile	Sterile Tap	No
3	Sterile	Non-Sterile Pond	No
4	Sterile	Non-Sterile Pond	Yes
5	Non-Sterile	Sterile Pond	No
6	Non-Sterile	Sterile Tap	No
7	Non-Sterile	Non-Sterile Pond	No
8	Non-Sterile	Non-Sterile Pond	Yes

The light measurements taken for this experiment were quantified by the LI-COR LI-1000 Data Logger using a small bubble sensor. A series of 12 representative light readings were taken inside the hood; six readings across the front and another six along the rear of the hood with the data logger set for instantaneous reading at one minute intervals. From these 12 individual readings, the average was shown to be $11.2 \mu\text{mol s}^{-1} \text{M}^{-2}$ per μA with a standard deviation of $0.48 \mu\text{mol s}^{-1} \text{M}^{-2}$ per μA .

Results and Discussion - Water Phase

For the nonsterile pond water and nonsterile TNT-containing soil containing Parrotfeather, the active bacterial biomass in water was higher than all other treatments. Further, the active bacterial biomass in all other treatments did not differ from the flasks containing sterile soil & sterile tap water as well as those containing nonsterile soil & nonsterile pond water without Parrotfeather. This data indicates that Parrotfeather plants promote bacterial growth in the water or serves as a source of inoculum.

Results and Discussion - Soil Phase

Analysis of the results show that Parrotfeather did not affect the numbers of bacteria in the soil. Also that

flasks containing nonsterile soil & sterile pond water without plants had more bacteria in the soil than flasks containing sterile soil & sterile pond water; sterile soil & nonsterile pond water with plants; and nonsterile soil & sterile tap water. Active bacterial biomass did not differ in all other treatments.

Most likely the nonsterile soil contained some bacteria that multiplied when sterile pond water was added. Sterile pond water would contain the necessary nutrients and carbon for bacterial growth without competition from predator organisms that might otherwise be contained in nonsterile waters.

Parrotfeather Effect on Aqueous Phase TNT Concentration

This laboratory experiment was designed as a concentration versus time study to determine the resulting TNT concentration in a TNT contaminated water after the addition of Parrotfeather plants. Overall, the experiment involved the preparation of a TNT contaminated water of known concentration, the addition of selected Parrotfeather plants, and finally the TNT concentration determination at preselected times.

Experimental Method

First, a grab sample of roughly 400 grams of the contaminated soil from the AAAP stockpile of the first field experiment was dried, crushed, screened, and thoroughly mixed. An elevated water TNT concentration was achieved by adding 100 grams of this prepared TNT contaminated soil to each of three one-liter glass flasks filled with deionized water. All flask mixtures were shaken vigorously and allowed to settle and equilibrate for two weeks. Afterwards, miscellaneous soil constituents and suspended particulate matter were removed from the TNT-water solutions by filtration through a Genuine American Grade 802 Filter Paper obtained from Fisher Scientific Supply. Then, the beakers of filtered TNT-containing waters were transferred to a five liter flask and thoroughly mixed to constitute the TNT contaminated experimental water. From this mixed water, ten 100 ml sub-portions were taken and placed into 250 ml beakers.

Next, five grams of the Parrotfeather was randomly added to five of the containers with the remaining five beakers serving as experimental controls. This achieved a 1:20 ratio of plant to water on a gram to milliliter basis.

All ten beakers were topped by aluminum foil to prevent excessive evaporation and were allowed to stand with no mixing. One ml subsamples were removed by pipette at

preselected times, then prepared and analyzed as detailed in the laboratory sampling section.

Results and Discussion

Two treatments with five repetitions were investigated; treatments were "with plants" and "without plants." Experimental results, sorted by beaker and sample time, are presented in Appendix D, Table D1 where the "without plants" treatments are lightly shaded for emphasis.

This data is summarized and averaged by treatment to yield a single data point from the averaged value for each of the five repetitions of each treatment. Averaged data is presented in Table 5.3 and visually depicted in Figure 5.1. From this data, the "with plants" treatment shows TNT removal from the water with a half-life of roughly twenty hours when exposed to the Parrotfeather at a ratio of 1:20 (weight of plants in grams to volume of water in milliliters). In about four days, the TNT concentration in the aqueous phase was reduced below HPLC detection limits, approximately 0.1 ppm.

After the 147 hour sampling, all plants were removed from the beakers containing them and the systems were set aside and left undisturbed for the remainder of the month.

Table 5.3

Parrotfeather Effect on the Aqueous Phase TNT Concentration

Sorted by Time and Treatment Method

(Page 1 of 2)

Time (hour)	Treatments	
	Without Plants	With Plants
	TNT (ppm)	TNT (ppm)
0.0	84.6	84.6
0.5	84.6	64.3
1.0	85.2	62.6
1.5	82.9	56.5
2.0	83.3	54.1
2.5	77.7	50.1
3.0	82.6	45.9
3.5	82.9	48.9
4.0	79.2	46.5
5.0	84.0	52.8
6.0	86.1	51.3
7.0	90.5	54.2
8.0	89.8	49.7
15	83.1	43.5

Table 5.3

Parrotfeather Effect on the Aqueous Phase TNT Concentration

Sorted by Time and Treatment Method

(Page 2 of 2)

Time (hour)	Treatments	
	Without Plants	With Plants
	TNT (ppm)	TNT (ppm)
26	89.4	35.1
30	88.0	30.4
37	87.0	22.6
50	85.7	9.8
61	91.0	5.6
77	97.8	0.6
101	82.3	0.0
147	86.6	ND

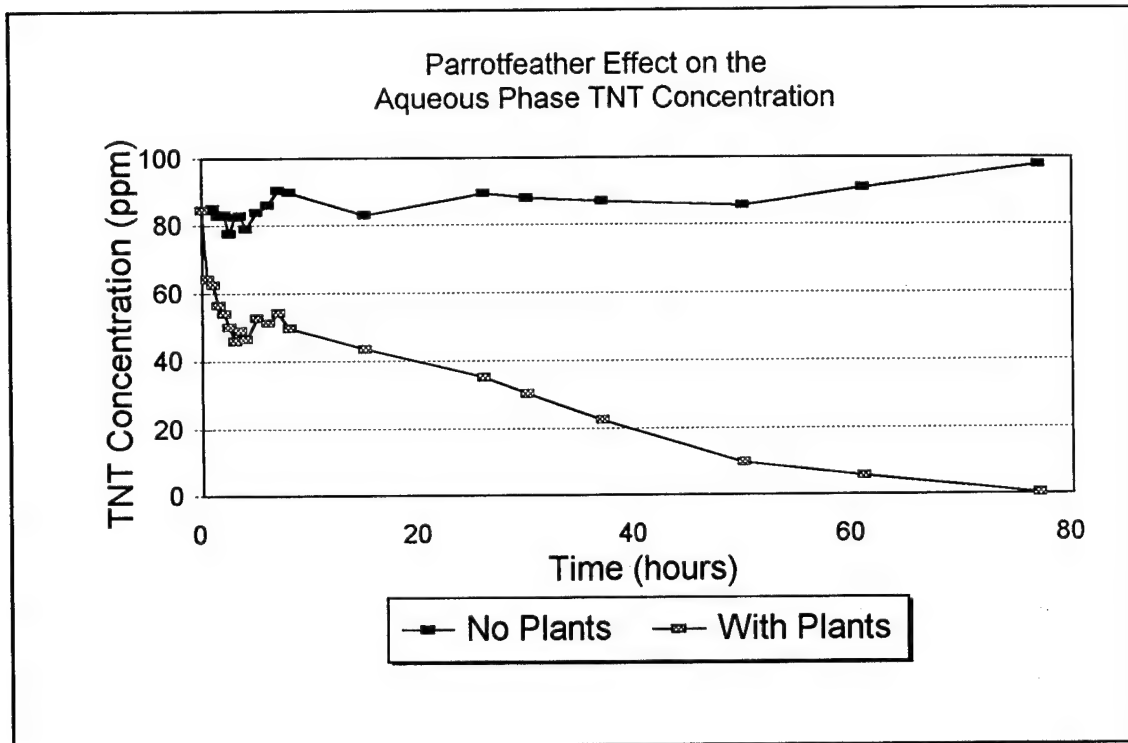


Figure 5.1

Parrotfeather Effect on the Aqueous Phase TNT Concentration

Final samples were taken thirty days after the initiation of the experiment and are tabulated at the end of Table D1. This shows that the non-plant water TNT concentration remains essentially unchanged over the thirty day period while the TNT was completely degraded below detection limits in the Parrotfeather incubated waters.

No product formation was quantified, but apparent product formation peaks were detected by HPLC analysis in the water containing Parrotfeather. These corresponded with the TNT monoamino and diamino reduction products retention times. Peaks at these retention times were not observed in the non-plant controls.

Further analysis of the "with plants" data shows that TNT degradation by Parrotfeather at a 1:20 ratio most closely conforms to first-order kinetics. A plot showing this was developed by least squares analysis using Quattro Pro and is presented in Figure 5.2 with the equation of the line and coefficient of determination annotated.

Parrotfeather Effect on Solid Phase TNT Concentration

Another experiment was designed to measure the actual degradation of TNT from a contaminated soil after water and Parrotfeather addition. Agitation was applied to each soil-water mixture at the beginning of each step in this experiment to enhance the diffusion of TNT into the water.

Experimental Method

Ten grams of the prepared laboratory soil detailed in the laboratory materials section was added to 250 ml flasks containing 100 ml of deionized water. A total of ten flasks were established; five were randomly selected to receive ten grams of Parrotfeather plants, the remaining five served as controls. Before plant addition, each soil-water flask was mounted to a beaker shaker and agitated at three shakes per second to increase the TNT mass transfer from the soil to the water. After a relatively short settling period, the plants were added and enough time was allotted for the plants to deplete the aqueous phase TNT contamination. All plants were removed during agitation and settling periods to prevent excessive plant abuse. This process was repeated for a total of four agitation cycles. All 10 flasks were agitated at the same time and all were subjected to the same environmental conditions.

Aqueous phase TNT concentration determinations were made at the start and stop point of each step in this experiment. Three aqueous samples of one milliliter each were taken from each beaker at each sample time to improve sampling accuracy, however, only the final soil TNT concentration from each beaker was determined for comparison

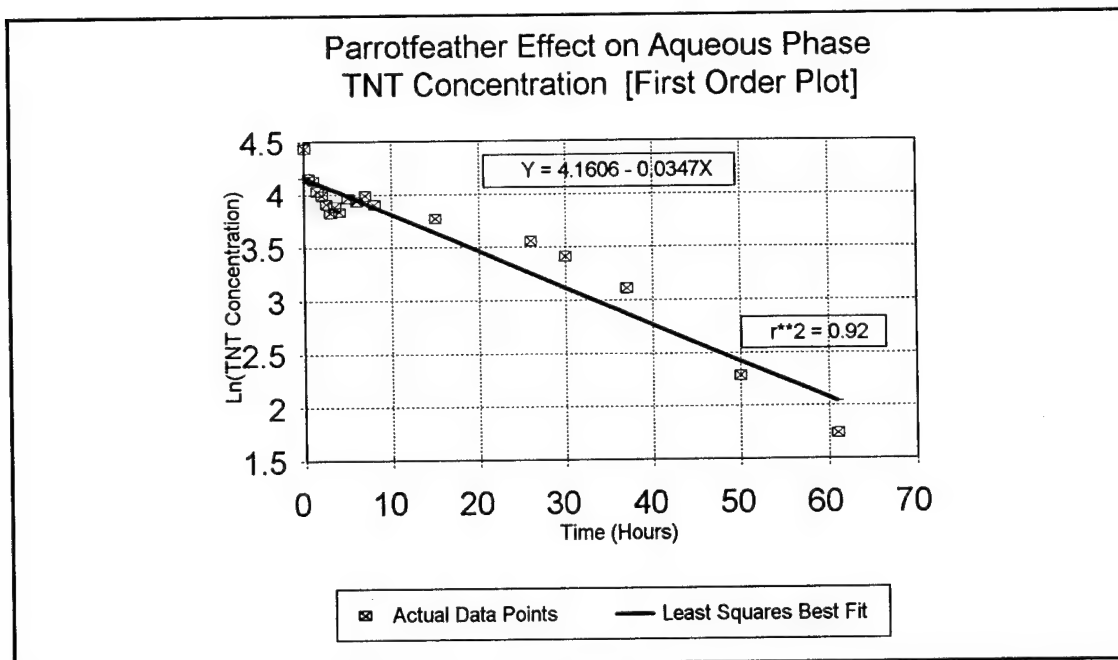


Figure 5.2
Parrotfeather Effect on Kinetics of Aqueous Phase TNT
Degradation

to the initial soil TNT concentration. All samples, both aqueous and solid, were prepared and analyzed as detailed in the laboratory sampling section.

A detailed listing of the experiment process by sequential step included:

- 1) Agitate the soil-water systems for four hours and then allow them to settle for 24 hours; samples taken after settling become step number 1.
- 2) Add plants to selected systems and allow them to equilibrate for 3 days; samples taken after equilibration become the first step number 2.
- 3) Remove the plants and agitate all systems for ten hours and then let them settle for 36 hours; samples taken after settling become the second step number 2.
- 4) Add plants again and allow the systems to equilibrate for 48 hours; samples taken then become the first step number 3.
- 5) Remove the plants and agitate the systems for 15 hours then let the systems settle for 3 days; samples taken now become the second step number 3.
- 6) Add plants again and allow systems to equilibrate for 48 hours; samples taken then become the first step number 4.
- 7) Remove the plants and agitate the systems for 16 hours then let the systems settle for 3 days; samples

taken after the final settling period become the second step number 4.

Results and Discussion - Aqueous Phase

All resulting aqueous sample data are sorted by process step and tabulated in Appendix D, Table D2. To better observe treatment trends, the data is summarized by step and averaged to yield a single data point for each of the five repetitions of each treatment. Synopsized data are sorted by treatment and presented in Table 5.4 with a corresponding visual depiction shown as Figure 5.3.

After the TNT was in the water, the Parrotfeather treatment consistently degraded the aqueous phase TNT, while the control remained roughly at a room temperature water saturation concentration. As the presence of Parrotfeater was the only treatment variable, the aqueous TNT degradation is directly attributable to Parrotfeather. The slightly elevated water TNT concentrations observed in this experiment likely resulted from suspended TNT-containing particulates caused by the agitation that were not completely settled by centrifugation.

Results and Discussion - Solid Phase

All resulting soil TNT concentration data are shown in Table 5.5. Analysis of this data by one-way ANOVA shows a

highly significant difference in the mean concentration of TNT between the treatments at the 95% level of significance.

When the combined total amount of TNT contained in soil and water is calculated and added, it is evident that the control "without plants" treatment did not exhibit a reduction in TNT concentration while the "with plants" treatment resulted in a high percentage of TNT degradation.

There is about 28 mg of TNT remaining in the ten grams of soil, and about 15 mg TNT in the 100 ml water for the control treatment. By simple addition, the control treatment at the end of the experiment contained on average 43 mg of TNT. When this is compared to the initial laboratory contaminated soil containing an average 44 mg of TNT in 10 grams of soil there was little change evidenced between the initial and final amounts of TNT in the control systems, at most a 2% decline.

On the other hand, the remaining average extractable TNT concentration for the ten grams of soil in the "with plants" treatment equates to about 2 mg of TNT. As there was no aqueous TNT concentration, the effective degradation of TNT by this treatment was around 91%.

Obviously, a significant agitation effort became necessary to enhance solubilization of the TNT from the soil into the water, however, Parrotfeather quickly reduced TNT once it was in solution. The key to greatly improve the

Table 5.4

Parrotfeather Effect on the Aqueous Phase TNT Concentration
in a Beaker With TNT Containing Soil

TNT Concentration (ppm)		
Process Step	Treatments	
	With Parrotfeather	Without Parrotfeather
1	170.5	154.0
2	1.2	148.0
2	41.9	143.2
3	13.6	148.0
3	46.0	158.7
4	0.0	149.7
4	0.0	148.0

Note: Each TNT concentration data value is an average of the five treatment repetitions.

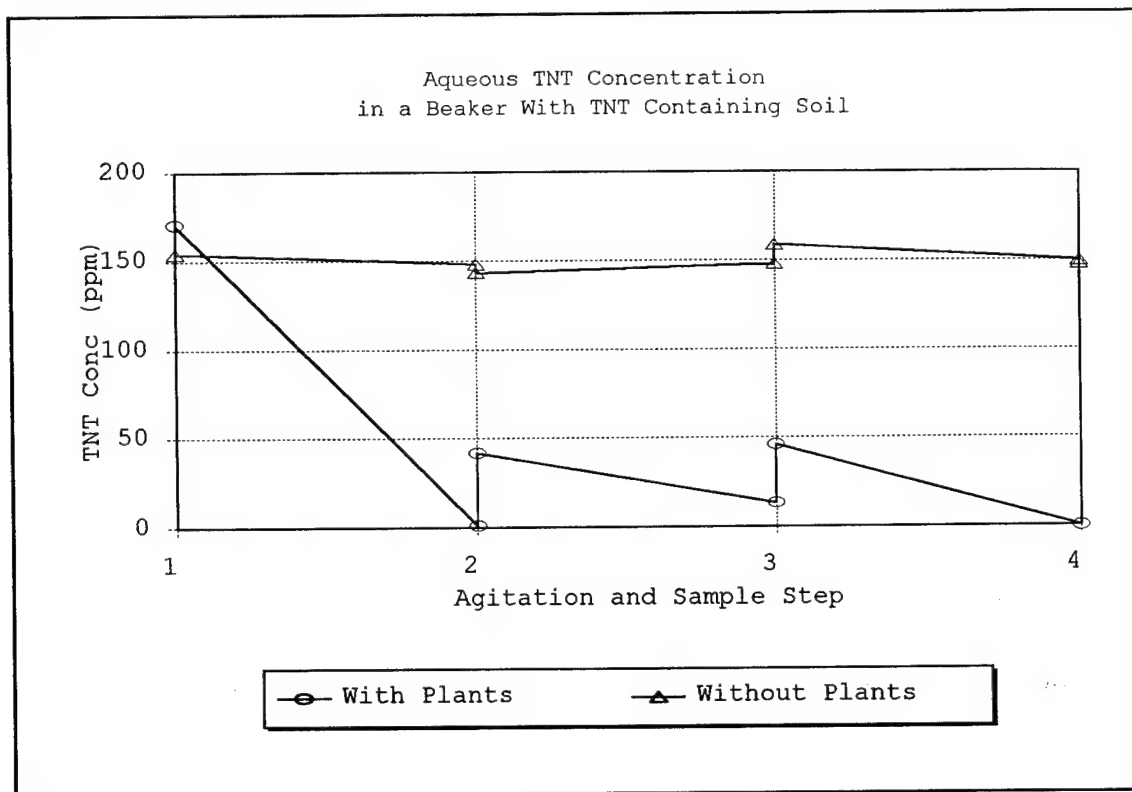


Figure 5.3

Aqueous Phase TNT Concentration in a Beaker With TNT
Containing Soil

Table 5.5

Parrotfeather Effect on the Solid Phase TNT Concentration -
 Soil TNT Concentration Remaining in the Experiment Beakers
 at the End of the Experiment and Sorted by Treatment Method

TNT Soil Concentration (ppm)		
	Treatments	
Beakers	With	Without
	Parrotfeather	Parrotfeather
1	104	2426
2	215	2511
3	230	2474
4	244	3029
5	295	3458
Average	218	2780

efficiency of Parrotfeather-TNT remediation is to increase mass transfer of the TNT out of soil and into the water.

Ten Week Soil-Water TNT Study

As the previous experiments demonstrated the capability of Parrotfeather to remove TNT from a contaminated media, a ten week laboratory experiment was undertaken to determine the effects of a batch process on the TNT removal from a contaminated soil by this plant. A batch mode process was selected to better model the previous field work conducted on-site at the AAAP.

Experimental Method

Similar to the previous laboratory study and emulating the field study, a contaminated environment was established by combining 10 grams of the prepared contaminated soil in 250 ml beakers with 100 ml of deionized water. A total of sixty beakers were established; half were randomly selected for plant addition while the remaining 30 beakers served as experimental controls. No agitation, other than initial materials addition, was used.

To ensure faster TNT removal, a 1:10 plant to water ratio was selected; 10 grams of plant for each 100 ml of water. After plants addition, each beaker was capped by aluminum foil to prevent excessive evaporation and placed

under a 40 watt four-foot fluorescent light fixture. The light source was left on continuously 24 hours each day.

Six beakers were randomly selected and dismantled for water and soil samples at each weekly sampling period. Three separate samples were taken from the aqueous phase of each of the six randomly selected beakers to improve the accuracy of the results. However, only one soil sample was taken from each system for the final soil TNT concentration measurement as the systems were dismantled. All samples, both water and soil, were prepared and analyzed as detailed in the laboratory sampling section.

Results and Discussion - Aqueous Phase

Resulting aqueous phase data are separated by treatment and presented in Appendix D, Table D3. A composite of the averaged concentrations sorted by week and treatment is shown in Table 5.6 and visually depicted by the plot in Figure 5.4. Some reduction in the aqueous phase TNT concentration that occurred in the experimental controls over the ten week period was likely due to settling of suspended materials containing TNT. However, little TNT was ever found in the water of the containers having plants over the course of the ten weeks, even in the early weeks of the experiment.

Table 5.6

Ten Week Study - Aqueous Phase TNT Concentration Averaged
and Sorted by Treatment

Averaged TNT Concentration (ppm)		
Time (Weeks)	Treatments	
	With Plants	Without Plants
0	0	0
1	0.3	152.8
2	0	156.2
3	0.3	116.5
4	0	117.7
5	0	127.4
6	0	110.9
7	0	116.0
8	ND	85.8
10	ND	90.4

Note: Each TNT concentration in this table is an average of nine samples; three water samples were taken from three beakers with three repetitions each.

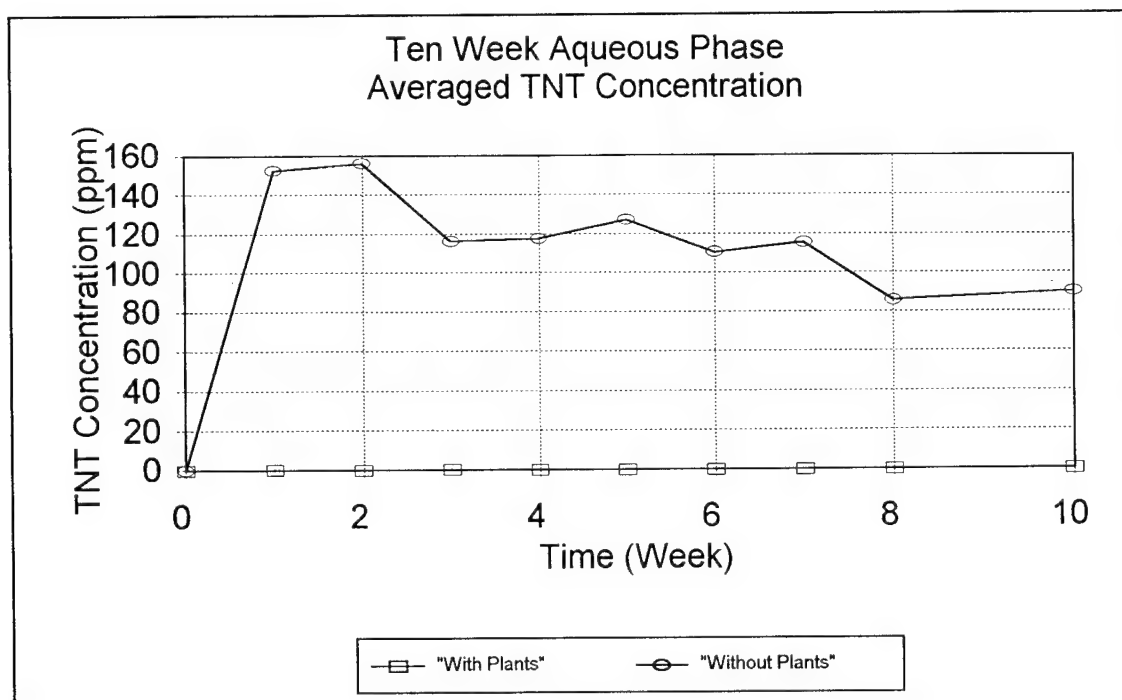


Figure 5.4

Ten Week Experiment Averaged Aqueous Phase TNT Concentration

Results and Discussion - Solid Phase

Each of the weekly soil TNT concentration determinations are shown in Appendix D, Table D4. Statistical analysis of these data by one-way ANOVA shows a difference in treatment means at the 95% level.

The remaining TNT concentration in the soil, sorted and averaged by treatment and week, is displayed in Table 5.7 with a corresponding plot shown as Figure 5.5. Although the plants began to darken between the second and third week and all were obviously dead by week five, there was a very obvious statistical difference between the treatments. Thus, the Parrotfeather treatment in this experiment, at the 1:10 plant-to-water ratio and in a batch mode of operation, does result in a significant soil TNT concentration decrease. A very thin layer of soil and the increased particle surface area due to the smaller particles of the prepared homogenous laboratory soil likely increased the mass transfer potential in the soil-water system.

As the same soil source was used in all beakers and the plant treatment beakers were chosen randomly, the degradation of TNT from the soil by Parrotfeather is evident from this experiment. The total milligrams of TNT remaining in each treatment, averaged per week, was calculated and listed in Table 5.8. Of great significance, the "with plants" treatment eliminated around 82% of the TNT from the

Table 5.7

Ten Weeks Soil-Water Study - Averaged TNT Concentration in
the Soil Sorted by Time and Treatment

TNT Concentration (ppm)		
Week	Treatments	
	With Plants	Without Plants
1	786.7	3007.3
2	630.7	2686.3
3	213.3	2675.3
4	221.3	2516.0
5	236.0	2844.7
6	172.7	2749.7
7	181.7	2493.3
8	156.3	2532.0
10	130.3	2903.0
Average	303.2	2712.0

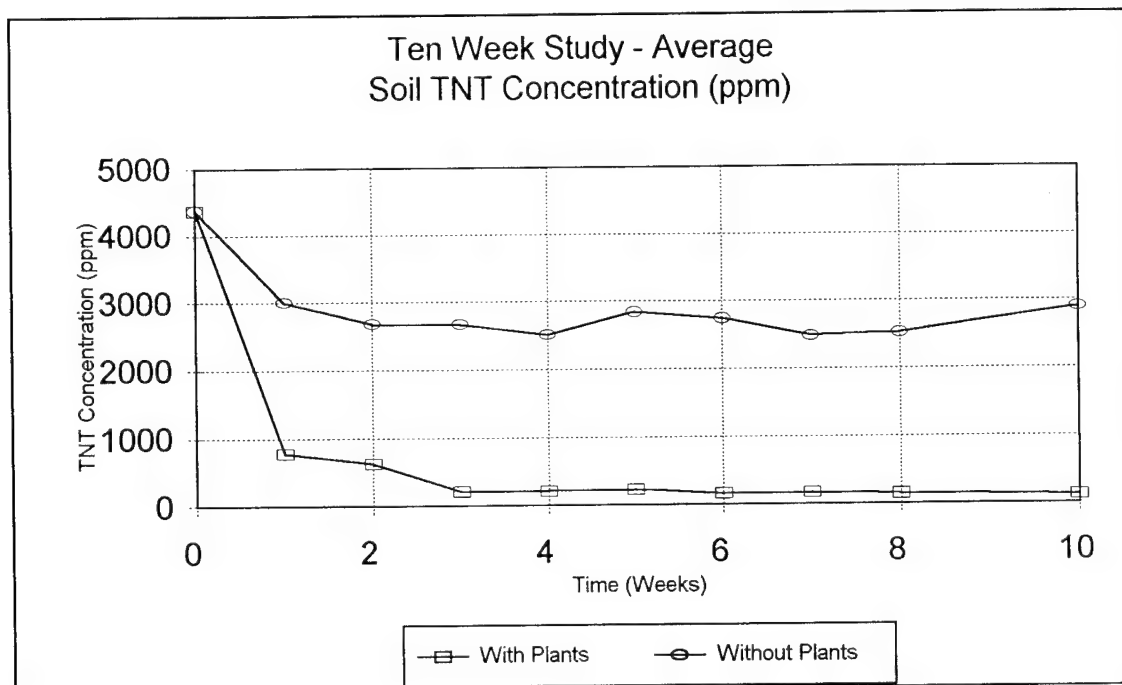


Figure 5.5

Ten Week Experiment Averaged Soil TNT Concentration

Table 5.8

Ten Week Experiment - Total Amount of TNT (mg) Sorted by
Treatment by Week and by Media (Water or Soil)

Week	Total Amount of TNT (mg)					
	With Plants Treatment			Without Plants Treatment		
	Water	Soil	Totals	Water	Soil	Totals
0	0	43.8	43.8	0	43.8	43.8
1	0	8	8	15.3	30	45.3
2	0	6	6	15.6	27	42.6
3	0	2	2	11.6	27	38.6
4	0	2	2	11.8	25	36.8
5	0	2	2	12.7	28	40.7
6	0	1.7	1.7	11.1	28	39.1
7	0	1.8	1.8	11.6	25	36.6
8	ND	1.6	1.6	8.6	25	33.6
10	ND	1.3	1.3	9	29	38

soil within the first week. By week ten, less than 3% of total TNT remained in the averaged "with plant" treatment even though the plants died. Conversely, no significant change was evidenced in the "without plants" treatment where 38 mg of TNT remained after ten weeks.

Aqueous Phase ^{14}C -TNT Fate in Parrotfeather and Water System

The purpose of this experiment was to determine the fate of the aqueous phase ^{14}C -TNT after the addition of Parrotfeather plants. A ^{14}C TNT-water solution was prepared and used to detect and quantify the decomposition of the labeled TNT compounds.

An attempt to identify the major breakdown components due to degradation of TNT by Parrotfeather was a significant feature in the design of this experiment. Also, it was designed to chart the species and concentration of the labeled products at each time interval.

Labeled TNT-Water Solution Preparation

A ^{14}C radiolabeled TNT solution was formulated by dissolving a small portion of ^{14}C ring labeled TNT detailed in the Chemicals and Standards section of Chapter Three into one ml of methanol. A 100 μl aliquot of this labeled solution was removed and combined with 10 ml of scintillation cocktail to form a sample for initial activity

determination. Liquid scintillation counting was used to determine the activity of this labeled solution; roughly 370,000 disintegrations per minute (dpm) in each 100 μ l sample.

An HPLC analysis was also performed to determine the TNT concentration of this solution. A sample was taken and diluted with acetonitrile until the TNT concentration could be determined. The TNT concentration of the original labeled solution was found to be about 10,000 ppm.

Experimental Method

As the labeled solution TNT concentration was well above water solubility limits, it was necessary to dilute this prepared solution by a factor of 100. The resulting TNT concentration was in the upper range of water solubility, roughly 100 to 120 ppm (Ou 1994b). Twenty ml of this solution was then added to five test beakers. Afterwards, four grams of thoroughly-washed Parrotfeather was added to each beaker yielding a 1:5 ratio; weight of plants in grams to volume of water in milliliters. These plant-water systems were allowed to equilibrate for five predetermined time intervals: 3, 12, 24, 48, and 73 hours. At each preselected time, a system was harvested and analyzed for TNT concentration and total ^{14}C activity.

Each system disassembly consisted of: removing the plants from the labeled solution, washing the plants with deionized water, freezing and crushing the plant tissues, extracting the TNT from the pulverized plant material, and finally separating the bulk plant fibers from the extraction liquid.

As the plants were removed from solution, any lingering labeled solution was gently removed with a deionized water wash. All the plant wash water was retained and later evaluated for residual activity.

Once washed, the Parrotfeather was placed in a mortar where liquid nitrogen was applied to flash freeze the plant tissues. After freezing, tissues were hand crushed by pedestal. Acetonitrile was added to the homogenized pulp material and thoroughly mixed. Afterward, the acetonitrile-pulp materials were transferred into four glass 15 ml screw top tubes, mixed by a vortex mixer, and then ultrasonicated. All four glass tubes containing the homogenized mixtures were centrifuged at 3,000 rpms for 15 minutes to separate the solid matter from the solution. After this, the solution was removed from each of the four glass tubes and placed into a single flask. Each tube, containing the plant material remains, were placed under an exhaust hood and allowed to dry completely.

The mortar was thoroughly washed with acetonitrile to remove residual labeled components once the plant pulp was removed from the mortar and placed into the glass tubes. This solvent wash was also stored and analyzed for radioactivity.

After the plant remains were thoroughly dried, they were removed from the glass tubes and placed in combustion cups. A combustion oxidizer was subsequently used to determine the radioactivity associated with the dried plant materials.

Radioactivity determinations were made at each individual pause point as the TNT-water-plant systems were dismembered. Thus, radioactivity determinations were made for the plant wash water, the mortar wash, the Parrotfeather incubated water, the plant-solvent extraction solution, and the dried plant fibers.

Two TNT concentration determinations were also made at each preselected time step. These were taken to observe the TNT concentration remaining in the water and the concentration in the extracted plant solution.

Analytical Method - ^{14}C -TNT Fate in Water

A one ml radiolabeled aqueous sample was taken from each beaker after the plants were removed and the ^{14}C -containing solution contents thoroughly mixed. This aqueous

phase sample was placed in a 1.5 ml plastic centrifuge vial and centrifuged at 14,000 rpms for 10 to 15 minutes. An aliquot was removed and diluted, as necessary, for subsequent TNT concentration determination by reverse phase HPLC.

Additionally, an undiluted 100 μ L aliquot was injected into the HPLC for separation and radio counting of the collected fractions. The radiolabeled waste stream leaving the HPLC column was captured in scintillation vials in 20 second increments for the first 15 minutes and each minute thereafter for an additional 15 minutes. Scintillation cocktail was then added and the samples were analyzed for radioactivity by liquid scintillation spectrophotometry.

Results and Discussion - ^{14}C -TNT Fate in Water

A table of the aqueous phase radioactivity (dpm) is included in Appendix D, Table D6. Further, comparison plots of the aqueous radioactivity obtained at two contiguous time intervals are included in Appendix D, Figures D1 through D4. These activity versus time of elution plots show the transformation of the labeled ^{14}C -TNT into radiolabeled products. It is apparent in Figure 5.6, showing the 3 hour

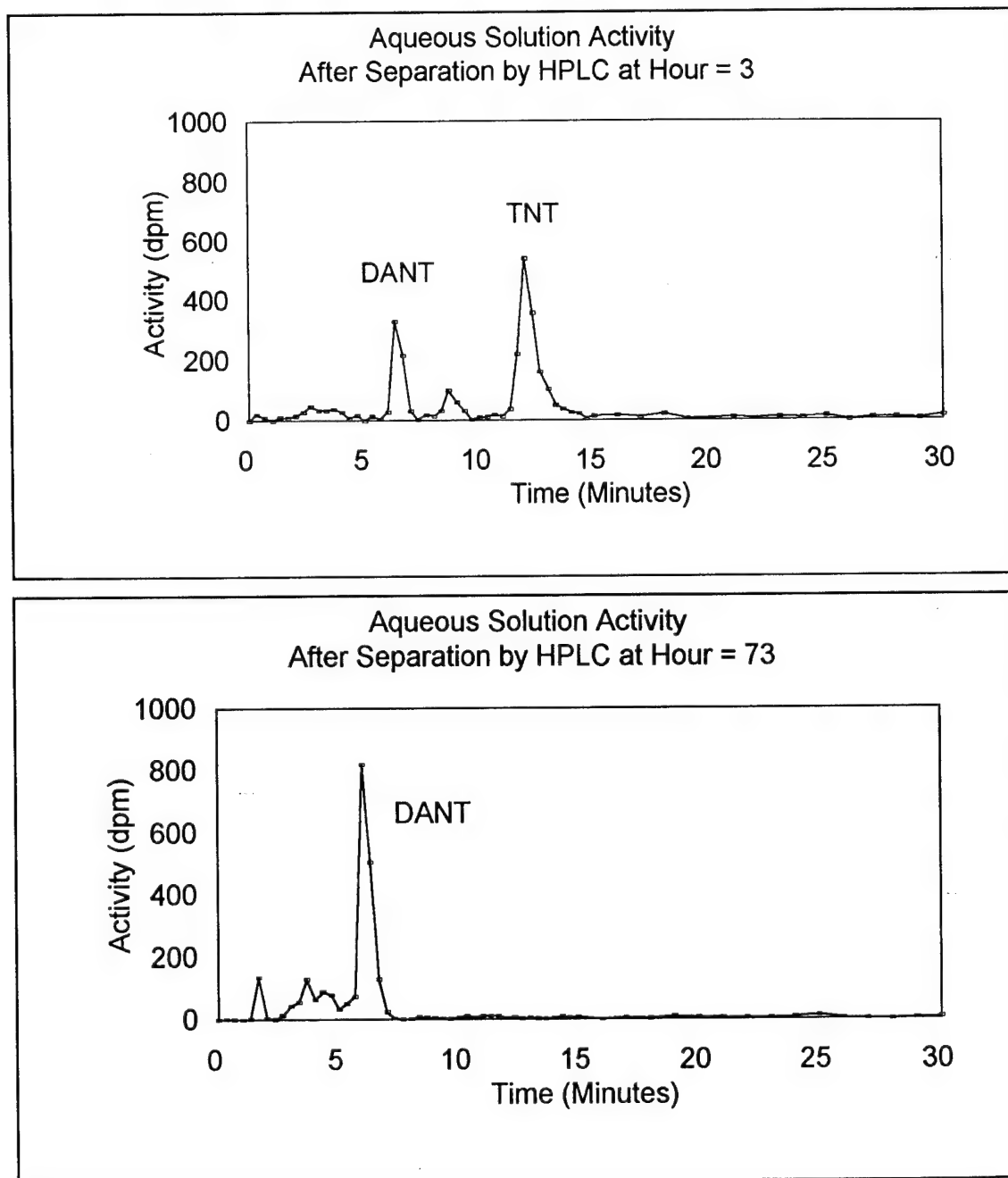


Figure 5.6

Comparison Plots of Aqueous Solution Activity, Hours 3 and

73.

analysis compared to the analysis at hour 73, that the ^{14}C -TNT observed in the water of the 3 hour sample has transformed mainly into ^{14}C -DANT.

GC/MS analysis of the 73 hour water sample confirmed the presence of 4A and both diamino isomers with the 2,4DA as the major product in this sample (Ou 1994b). An annotated GC/MS chromatogram developed from this sample is shown as Figure 5.7.

Analytical Method - ^{14}C -TNT Fate in Parrotfeather

A 1.5 ml aliquot was removed from the homogenized and extracted plant-solvent solution by pipette after the plant solid materials were removed. This extracted plant-solvent sample was placed in a 1.5 ml plastic centrifuge vial and centrifuged at 14,000 rpms for 10 to 15 minutes. A clarified aliquot was taken and diluted, as necessary, for subsequent TNT concentration determination by reverse phase HPLC.

Additionally, an undiluted 100 μL aliquot of the ^{14}C -TNT extracted plant-solvent was injected into the HPLC for radioactivity separation determinations. Identical to the aqueous activity separations, the solvent waste stream leaving the column was captured every 20 seconds for the

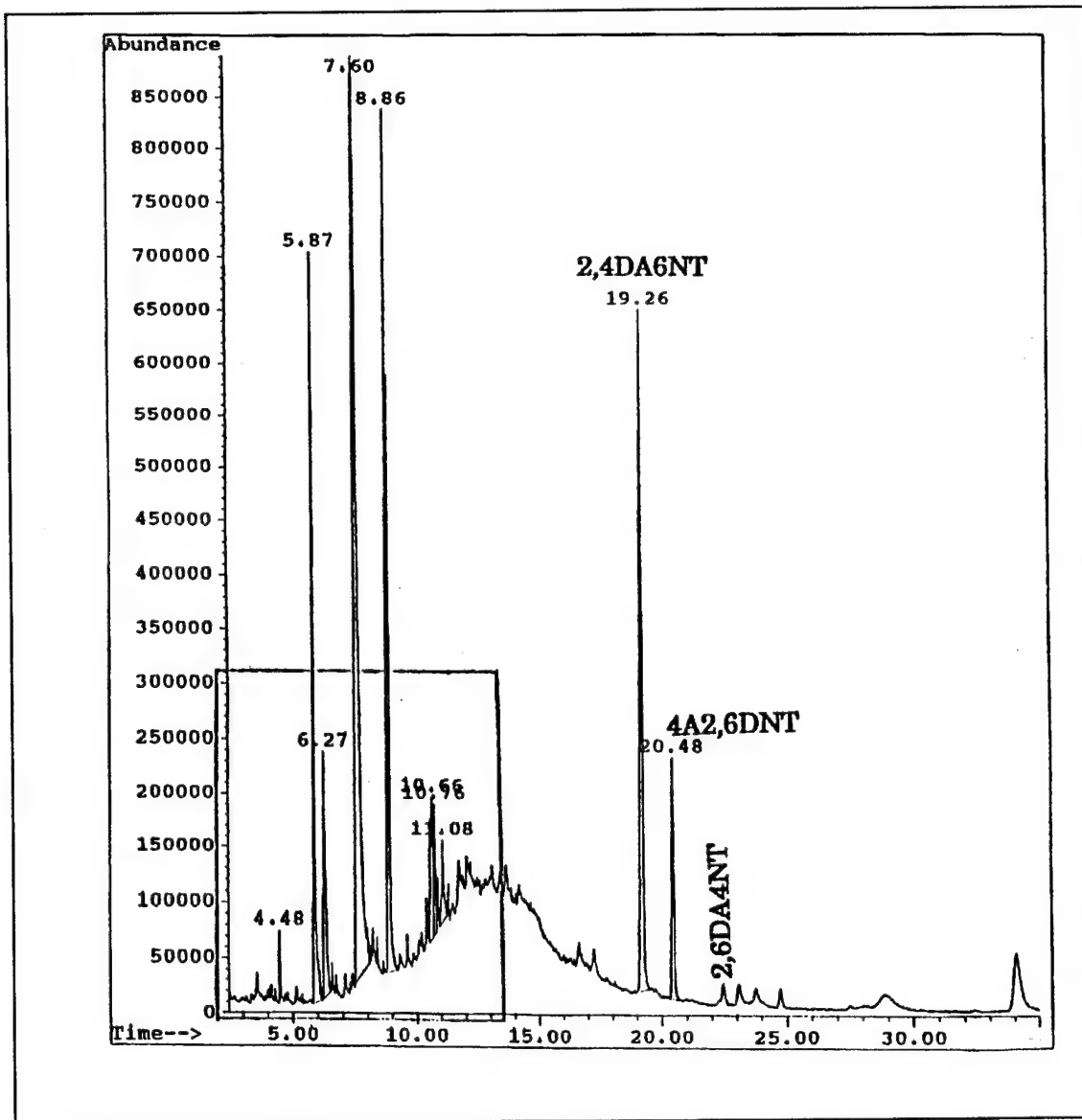


Figure 5.7

GC/MS Chromatogram of the Aqueous Phase Fate of Radiolabeled TNT Showing the Major Reduction Products After Incubation With Parrotfeather For 73 Hours

first 15 minutes, and once for each 15 minutes thereafter for activity analysis.

Results and Discussion - ^{14}C -TNT Fate in Parrotfeather

A complete listing of the extracted plant radioactivity (dpm) is detailed in Appendix D, Table D7. Further, comparison plots of the activity obtained from the extracted plant solution at two contiguous time intervals are included in Appendix D, Figures D5 through D8. These show a steady transformation of the ^{14}C -TNT associated with extracted plant materials into radiolabeled products analogous to those of the aqueous phase. However, the radioactivity was significantly lower than was found in the aqueous phase. As the radiolabeled amino reduction products are very water soluble, this is not surprising.

As a overall comparison, Figure 5.8 shows the 3 hour analysis compared to the 73 hour analysis. It is seen from this plot that the ^{14}C -TNT observed in the 3 hour sample has been completely transformed into products.

Summary

A summary of the ^{14}C activity separated by major component is included as Table 5.9 and plotted as Figure 5.9. Good radioactivity recovery was obtained at each step

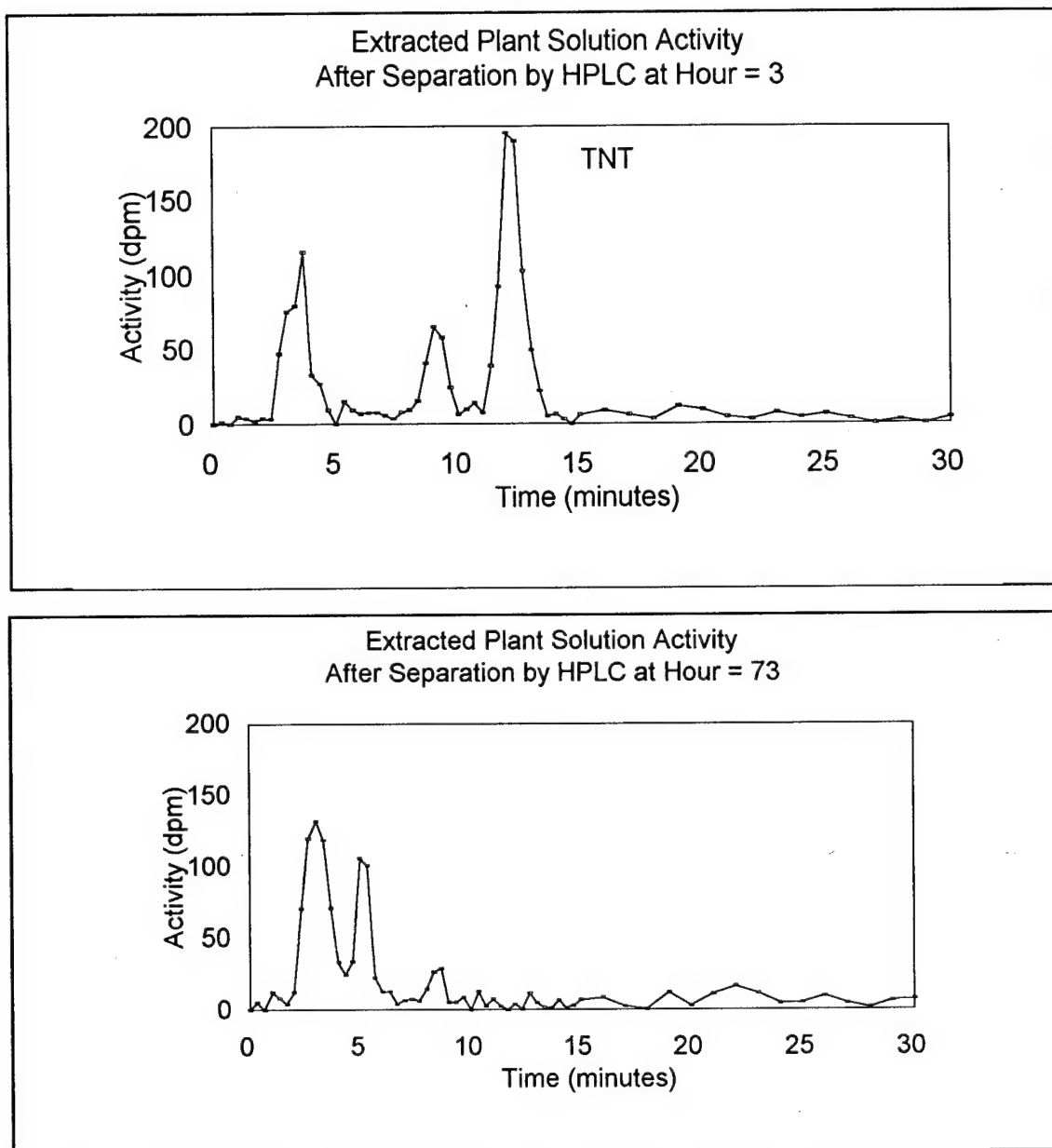


Figure 5.8

Comparison Plots of Extracted Plant Solution Activity, 3 and
73 hours.

Table 5.9
¹⁴C Activity in Each Major Experimental Component of the TNT-Water System After
 Incubation With Parrotfeather

¹⁴ C Activity in Each Major Experimental Component After Incubation With Parrotfeather						
Time Hours	Total Calculated (dpm)	Plant Wash (dpm)	Aqueous Phase (dpm)	Plant		
				Solution (dpm)	Oxidation (dpm)	Total (dpm)
0	1472800	0	0	0	0	0
3	1554699	359560	586600	452400	156139	608539
12	1479467	347550	519400	399600	212917	612517
24	1540194	281800	482000	458700	317694	776394
48	1496121	147550	546400	436800	365371	802171
73	1299500	163800	413000	357300	365400	722700

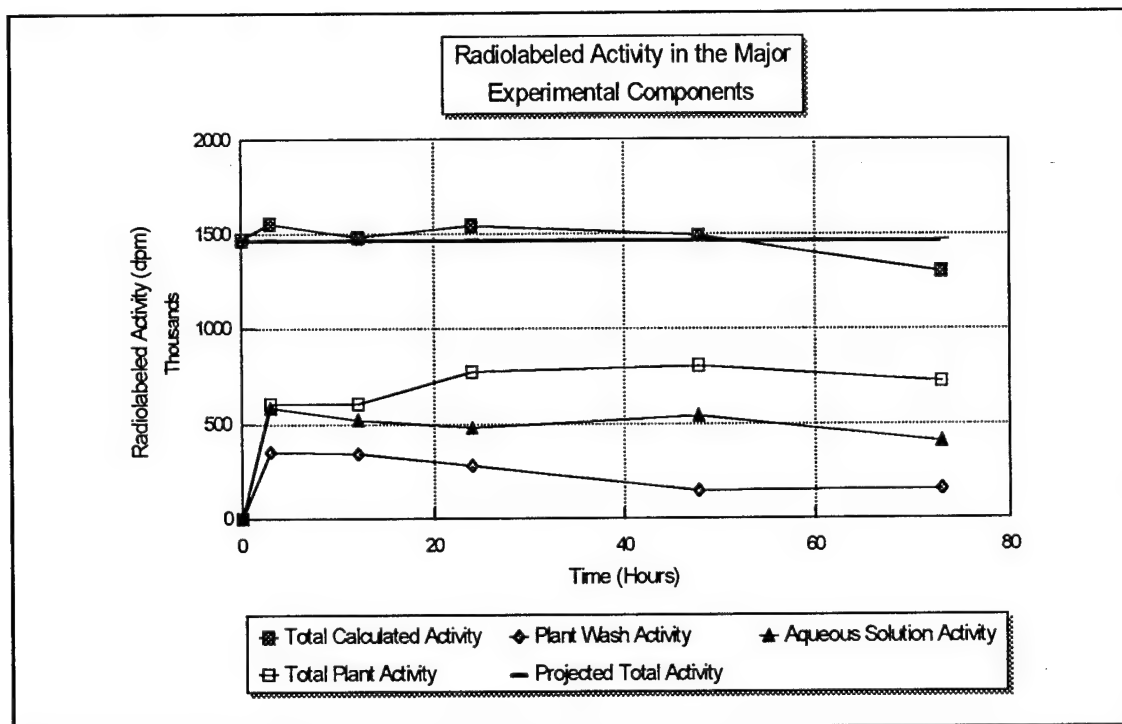


Figure 5.9

^{14}C Activity in Each Major Experimental Compartment of the
TNT-Water System After Incubation With Parrotfeather

indicating that the applied method properly accounted for all radioactivity components. The recovery percentages obtained from each experimental component, at each time step, are detailed in Appendix D, Table D5 and show adequate recovery at each time step. Further, the labeled percentage attributable to $^{14}\text{CO}_2$ was less than 1% at the 73 hour time and is not considered a significant sink of labeled material.

No fate determination was performed for nitrogen. However, once the ring is opened, it is thought that nitrogen and carbon are incorporated into the plant tissues.

A table and corresponding plot of the overall TNT concentration versus time is included as Table 5.10 and plotted in Figure 5.10. These show that TNT is quickly removed from water with little TNT buildup associated with the extracted plant materials i.e. no sorption.

Also, this experiment resulted in the identification of the major breakdown components generated from the natural degradation of TNT by Parrotfeather; MANT and DANT. A series of plots detailing the activity over time, after separation by the HPLC, shows this sequence of labeled TNT reduction into labeled products. Because the same major reduction products are generated by both Parrotfeather and Stonewort, it is likely the Parrotfeather-TNT reduction

Table 5.10

Total TNT Concentration in the Aqueous Phase and Extracted Plant-Solvent Solution After Incubation With Parrotfeather

Time (Hours)	Aqueous Phase TNT Concentration (ppm)	Extracted Plant- Solvent TNT Concentration (ppm)
0	130	0
3	17	10
12	0.5	0.7
24	0	0
48	0	0
73	0	0

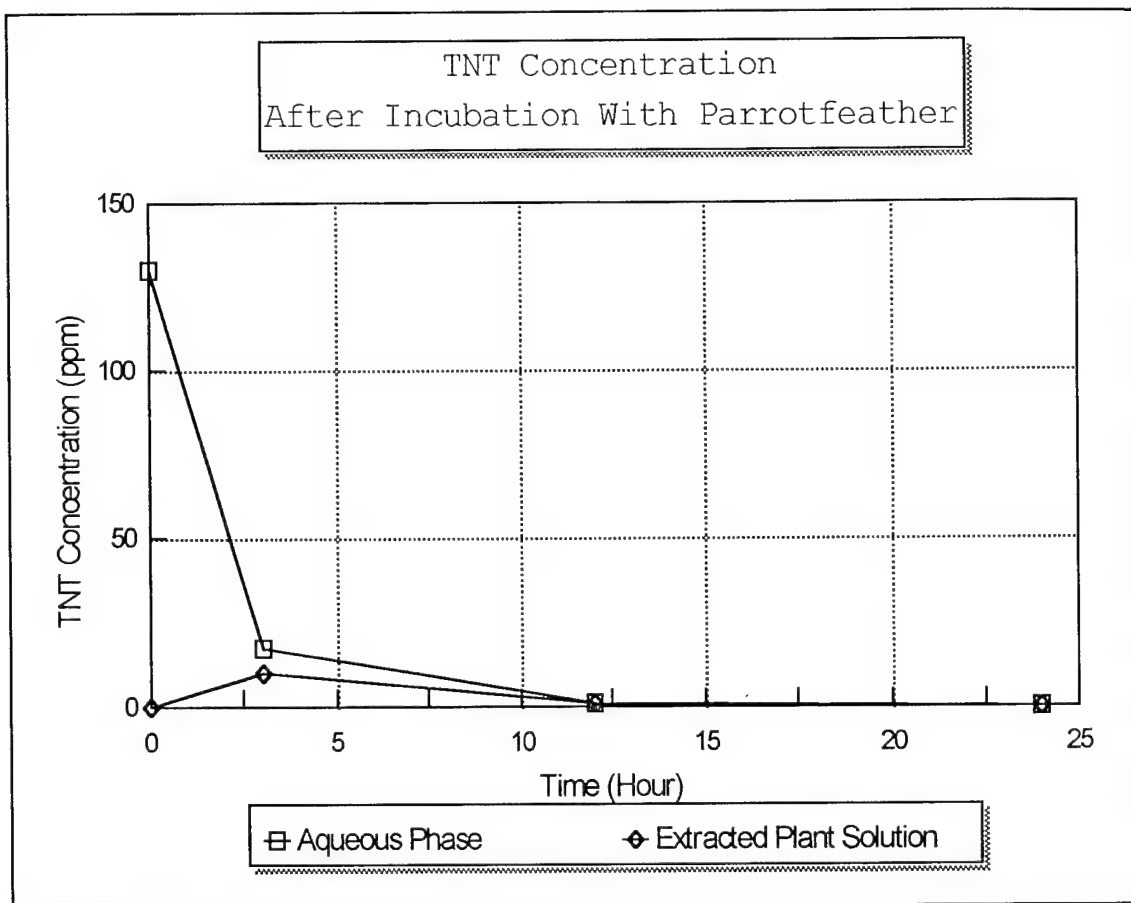


Figure 5.10

Total TNT Concentration After Incubation With Parrotfeather

pathway is similar to the proposed pathway for the aquatic plant Stonewort (Ou et al. 1994).

Water Incubated With Parrotfeather Plant-Free or Secreted Enzymatic TNT Reduction Study

A pond water analysis by the ERL-A found that half the nitroreductase activity of the Parrotfeather plant was also contained in the fresh pond water (Carreira 1994). These enzymes could be present in the pond water due to secretions or they may be released into the water when plants die and decompose. In either case, it could become important to know if TNT transformations occur due to nitroreductase enzyme activity in water and external to the plant. A simple experiment using deionized water and Parrotfeather plants was developed and executed to better illuminate this process.

Experimental Method

Ten grams of Parrotfeather was added to six 250 ml beakers containing 100 ml deionized water and allowed to incubate for three days. Then, the plants were removed from three of the beakers and 100 ml of a TNT-water solution of known TNT concentration was added to all six beakers. Measurements of the resulting TNT concentration were

Table 5.11

TNT Concentration in the Aqueous Phase Measured Over Time
 Resulting From Treatments of Water Incubated With
 Parrotfeather Plants Versus Parrotfeather Plants

(Page 1 of 2)

Averaged TNT Concentration (ppm)		
Time (Hours)	Treatments	
	Parrotfeather- Incubated Water	Parrotfeather Plants
0	60.0	60.0
0.25	53.0	46.6
0.5	51.4	41.5
1	54.4	44.1
1.5	52.5	38.5
2	44.9	36.3
2.5	52.7	37.3
3	45.5	36.3
3.5	46.4	34.3
4	51.9	37.2

Table 5.11

TNT Concentration in the Aqueous Phase Measured Over Time
 Resulting From Treatments of Water Incubated With
 Parrotfeather Plants Versus Parrotfeather Plants

(Page 2 of 2)

Averaged TNT Concentration (ppm)		
Time (Hours)	Treatments	
	Parrotfeather- Incubated Water	Parrotfeather Plants
5	46.3	27.7
6	43.0	26.0
7	54.0	25.3
22	40.4	6.9
25	42.7	5.4
31	42.7	2.9
45	37.2	0.1
54	44.0	ND
93	48.0	ND

obtained at preselected times and quantified by reverse phase HPLC analysis over a two day period.

Results and Discussion

A tabulation of the resulting data is presented in Appendix D, Table D8. This data is sorted and averaged by treatment then included as Table 5.11. The data shows that the deionized water and plant incubated solution only eliminates roughly one third of the TNT over the course of the experiment.

Because the pond water is known to contain nitroreductase enzymes and because deionized water incubated with Parrotfeather for three days demonstrates some TNT degradation, it can be inferred that the pond water used in the field experiments provided aqueous TNT reduction. Likely, this accounts for the amino reduction products found in the aqueous phase of the controls in the field experiment where the water source was the beaver pond water.

Plant, Plant Water, and Axenic Plant Comparison

There was a concern that organic particulates, remaining in suspension after plants removal, could bias the data obtained in the previous experiment. Therefore, the main purpose of these experiments was to repeat the previous experiment after eliminating any suspended particulates.

However, the scope of the experiment was expanded to also determine the TNT concentration reductions in a TNT-water solution from both nonsterile and axenic Parrotfeather plants. This experiment was conducted at the ERL-A (Carreira 1994).

Experimental Method

Ten grams of Parrotfeather was incubated for three days in 100 ml of deionized water. Then, Parrotfeather plants were removed and suspended particulates were removed from the water by filtration through glass wool followed by filtered water centrifugation at 14,000 rpms for 10 minutes. A protein assay using the standard MicroBCA Protein Assay Reagent Kit purchased from Pierce, Rockford, Illinois by the ERL-A did show that protein was present in the glass wool filtered TNT exposed-water samples.

For the other treatment comparisons, nonsterile Parrotfeather plants were prepared as previously outlined for laboratory plants and the axenic plant was removed from its sterile environment by Dr Carreira.

Each of the three treatments were inoculated with 2 ml of a TNT-water solution. A relatively low TNT concentration was used to decrease the time required to complete the experiment. Samples were taken over the next 24 hours.

Results and Discussion

The resulting data are presented in Table 5.12 with a corresponding plot included as Figure 5.11. All three treatments evidenced reductions in TNT concentration.

Although the plant incubated water reduced the TNT concentration below detection limits, the previous experiment shows that its reduction potential is limited. Likely if the low initial concentration had been higher, detectable TNT would have remained in the water of this treatment. Each distilled water sample that was incubated with Parrotfeather for three days contained nitroreductase, however, observations made by Dr. Carreira indicate that the nitroreductase activity in the plant incubated water was likely released into the water as the plants began to die. Regardless, because the plant incubated water showed TNT concentration reductions, the previous experiment was validated.

Also, the TNT concentration data for the axenic plant treatment would have likely been considerably lower if the plant had been submerged in the TNT-water solution; only

Table 5.12

TNT Concentration in the Aqueous Phase With Time From
Treatments of Parrotfeather Plants, Parrotfeather-Incubated
Water, and Axenic Parrotfeather Plants

TNT Concentration (ppm)					
Time (hours)	Plant	Time (hours)	Plant Water	Time (hours)	Axenic Plant
0.25	2.6	0.25	3.2	0.25	4.9
0.5	1.6	0.5	3	0.5	4.6
0.75	0.6	1	2.1	0.75	4.7
2	0.1	1.5	1	1	2.2
18	ND	2	1.2	1.5	1.5
24	ND	2.5	1.2	2	1.6
		3	1.2	2.5	1.4
		18	0	18	1.5
		24	0	24	1.2

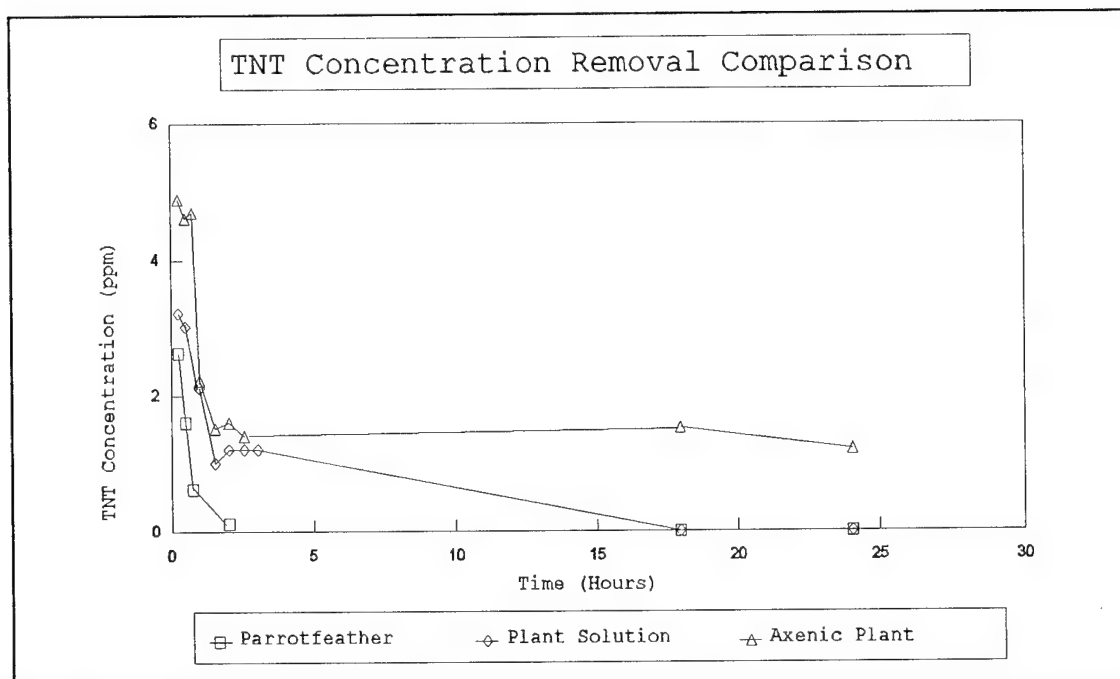


Figure 5.11

TNT Concentration in the Aqueous Phase Resulting Over Time
From Treatments of Parrotfeather Plants, Parrotfeather-
Incubated Water, and Axenic Parrotfeather Plants

about one third of the plant was actually in contact with the small volume of solution. This experiment with axenic Parrotfeather shows conclusively that the Parrotfeather plants cause reduction of the TNT in the water.

Nitroreductase Activity in the Parrotfeather Plant

The purpose of this experiment was to determine the specific part of the Parrotfeather plant having nitroreductase enzymatic activity: root, leaf, and stem. A protein MicroBCA Assay was conducted at the ERL-A by Dr. Carreira on Parrotfeather plant parts separated into stems, leaves, and roots. Resulting data shows the greater amount of protein to reside in the stems.

A subsequent nitroreductase assay by the Double Antibody method shows a corresponding greater amount of nitroreductase detected in the stems than in the leaves or roots. A chart detailing the results of this assay are included as Figure 5.12 (Carreira 1994). This data indicated that Parrotfeather contains nitroreductase, with the greater concentration located in the stems.

Effect of Plant-to-Water Ratio on TNT Reduction

The purpose of this experiment was to observe the TNT concentration reductions in water resulting from varying

Nitroreductase in Parrot Feather Double Antibody Assay

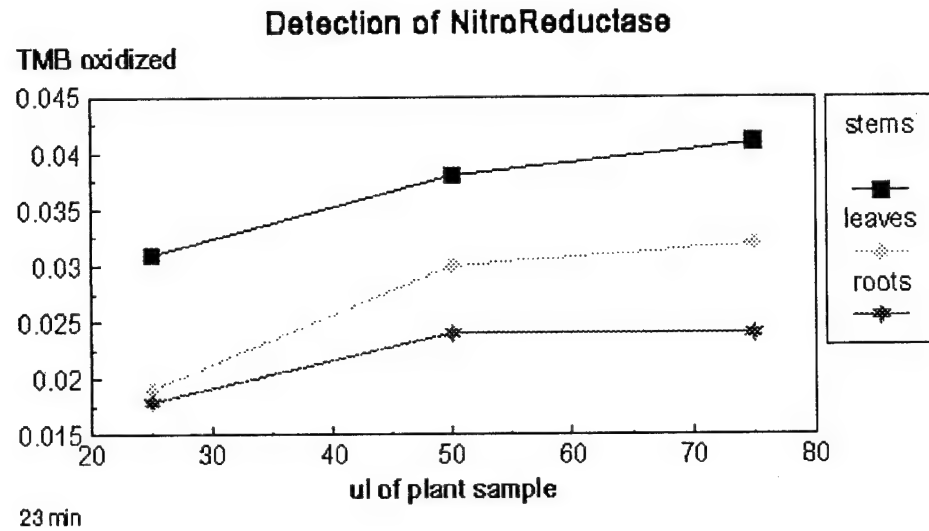


Figure 5.12

Nitroreductase in Parrotfeather - Double Antibody Assay

plant to water ratios. General observations of previous data indicates that the quantity of plants in the system directly affects the TNT degradation rate. Experimental results show that the TNT half-life is about one hour when in water with Parrotfeather at a 1:5 ratio. However, the half-life increases to about two hours when the plant to water ratio is decreased to 1:10 and twenty hours at a plant to water ratio of 1:20.

Experimental Method

A series of nine beakers, each containing 100 ml of TNT-contaminated water were set up. Parrotfeather was added in three different ratios of three repetitions to the beakers; blotted dry weights of one, two, and ten grams. This gave weight of plants in grams to volume of water in milliliter ratios of: 1:100, 1:50, and 1:10. These ratios were selected to expand the data base as the 1:5, 1:10, and 1:20 ratios were used in other experiments. The TNT concentration reductions in the water were observed over time.

Results and Discussion

A table incorporating the experimental results is included in Appendix D, Table D9. This data, averaged by treatment and tabulated by grams of plants to ml water, is

shown as Table 5.13 and plotted in Figure 5.13. Estimates for the TNT half-life at each ratio are: 2 hours at 1:10, 6 hours at 1:50, and 50 hours at 1:100. Also, at a 1:10 ratio the TNT is reduced below detection limits within 30 hours. However, reducing the ratio radically slows the reduction of TNT as shown in Figure 5.13. Only 80% of the TNT at the 1:50 ratio is gone in the first 48 hours with a small amount still present on day four. Although the 1:100 ratio shows some TNT concentration reduction, it is very slow with only around 60% gone in eight days.

Least squares analysis shows that the kinetics most closely resemble first-order. A first-order plot was developed for each plant-to-water ratio showing the actual data points and the calculated best fit line. These are included in Appendix G, Figures G1, G2 and G3. A composite of these plots, without the actual data points, is shown as Figure 5.14. As previously observed, the greater the ratio of plant-to-water, the faster the TNT is degraded as evidenced by the steepness of the plotted slopes.

Field Water Toxicity

No aquatic organisms were ever found in any of the "without plants" field waters although snails and tadpoles were observed to proliferate in the "with plants" waters. This laboratory experiment was conducted to determine if the

Table 5.13

Aqueous Phase TNT Concentration Resulting From the Varying
Ratio of Parrotfeather to Water

Time (Hour)	Parrotfeather to Water Ratio (grams of plant to ml of water)		
	1:100	1:50	1:10
0	119	119	119
0.5	90	95	56
1.0	109	95	61
1.5	96	95	59
2.0	109	69	55
2.5	102	89	65
3.0	86	72	47
3.5	74	59	24
4.0	108	68	43
5	77	55	29
6	64	64	29
21	56	18	4
24	77	33	1
30	86	32	ND
44	76	17	ND
53	55	14	ND
94	52	7	ND
192	44	ND	ND

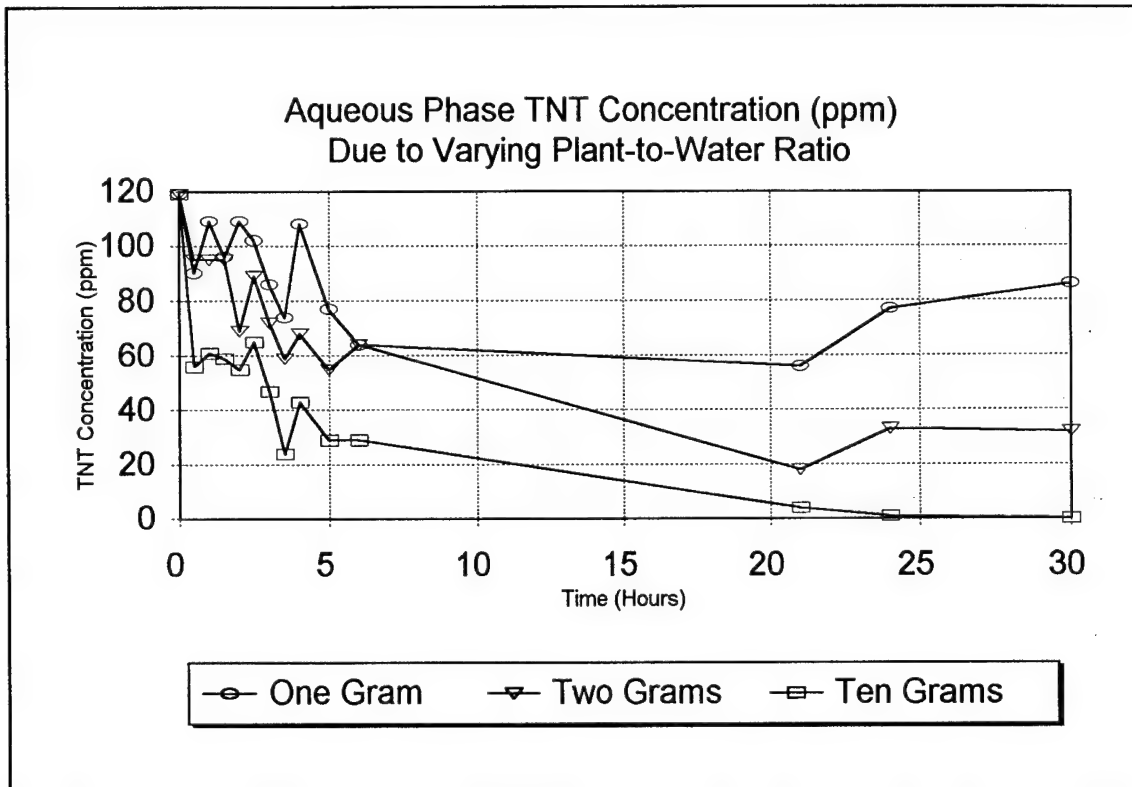


Figure 5.13

Aqueous Phase TNT Concentration Due to Varying Plant-to-Water Ratio (gm plants to ml TNT-contaminated water)

water from the non-plant treatments would support a representative sample of these aquatic organisms.

Methods and Materials

Tadpoles were selected as the test subject because they were the most numerous and easiest to acquire aquatic organisms in the field container waters. At the conclusion of the second field experiment and before the field containers were drained and dismantled, a number of tadpoles were captured from the "with plants" treatment containers. At the same time, one liter field water samples were taken from three individual containers of each treatment; a total of six samples, three turbid but clear and three burgundy colored.

In the laboratory, 650 ml aqueous subsamples were taken from each of the field container water samples and placed into individual one liter flasks. Four tadpoles were randomly selected and placed into each liquid sample for observation. The flask were not covered to allow for air exchange.

Observations

A check four hours after their transferral into the samples of the field waters revealed that all twelve tadpoles placed in the three burgundy colored "without

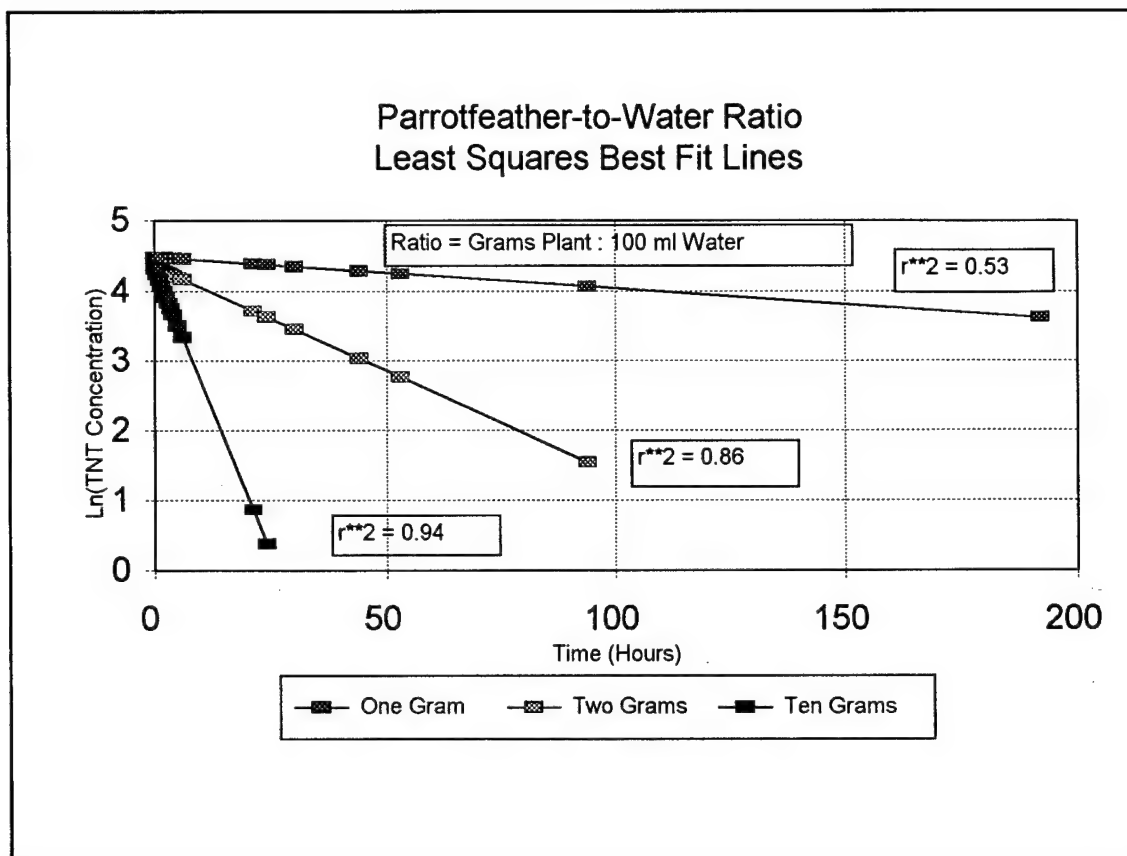


Figure 5.14

Calculated Least Squares Best Fit Lines for Varying Ratio of
Parrotfeather-to-Water (Data From Table 5.13)

plants" waters were dead. Tadpoles in the "with plants" waters were visibly unaffected and were very active.

These remaining tadpoles in the "with plants" waters were found to be alive and vigorous over the next 54 hours. It was not until one week after their transport to the laboratory and addition to the field waters that four of these tadpoles were found dead. Two of the deaths occurred in one container and one in each of the other two containers. However, only four tadpoles remained alive and vigorous in the containers; the other four had escaped from the glass containers.

Each of the tadpoles found dead at the weeks end had four legs and two had lost their tails; the other two only had a small stub of tail remaining. It is possible that these deaths resulted from a lack of nourishment and an inability to escape from the confining containers. After this week of observation, the remaining tadpoles were removed from the "with plants" treatment waters and placed in a beaker containing fresh pond water and Parrotfeather. After further maturing, they were tentatively identified as a variety of toad, probably a Southern Toad.

Discussion and Conclusions

Observations from this tadpole and field water experiment reveal the burgundy colored waters to be acutely

toxic to the tadpoles. Additionally, unidentified aquatic insect larvae were observed in the "with plants" field treatment containers but not in the non-plant treatment containers. Thus, it is plausible that the colored water is toxic to other organisms.

The greatest difference between the two field treatment waters was Parrotfeather addition to one and increased exposure to sunlight with elevated TNT concentrations in solution in the other. Possibly, a photolytic decomposition product of the TNT in the non-plant treatment water or the dissolved TNT itself is the agent toxic to these aquatic organisms. Apparently, Parrotfeather addition enhanced the aquatic environment for these organisms by removal or inhibition of the component(s) generating the harmful effects.

CHAPTER 6

SUMMARY AND RECOMMENDATIONS

Summary

This research demonstrated the degradation of TNT in bench-scale field experiments by Parrotfeather. Parrotfeather was found to have a nitroreductase enzyme capable of promoting the nitro group reduction of the TNT compound to amino groups. Experimental results show the complete nitro reduction of TNT, from aqueous phase TNT to the mono-amino isomers through the di-amino isomers, to the final amino reduction product, triaminotoluene. Afterwards, further actions by the plant, microbial activities, and/or autooxidation, result in ring opening and the ultimate mineralization of the TNT compound.

Significantly, both TNT and the potentially hazardous amino reduction products were degraded. No buildup of TNT or amino products was observed in waters of the field or laboratory experiments when Parrotfeather was present. Any initially detectable quantities were quickly decreased below detection limits although a time lag was seen in the final decline of the amino reduction products. Thus, a

significant feature of the Parrotfeather treatment not found in most other bioremediation schemes is the direct elimination of the hazardous amino products from the water in contact with the plants. Also significant, this plant system continues to flourish and to degrade TNT during a central Alabama winter.

TNT degradation by Parrotfeather occurs rapidly when the TNT is in water, although the TNT half-life is strongly plant-to-water ratio dependent. At a 1:5 ratio, measured in grams of Parrotfeather to milliliter of water, the half-life of TNT is approximately one hour. When the ratio is reduced to 1:20 the half-life increases to about twenty hours. The reaction kinetics were first-order with increasing plant concentrations resulting in higher rates of TNT degradation.

Although TNT solubility is known to be a function of temperature, the low aqueous phase TNT concentrations during summer indicates that temperature was not the controlling step. Apparently, mass transfer is the rate limiting step. This was evidenced by the relatively low aqueous phase TNT concentrations encountered in the field experiments. Also the laboratory experiment on the Parrotfeather effect on the solid phase TNT concentration required extensive agitation to speed up the diffusion process for TNT from the soil into the water.

Further evidence of the mass transfer limitations was uncovered during the ten week experiment. Rapid and significant TNT reductions occurred in the contaminated soil when the homogenous, finely ground, and very thin layer of soil provided a situation better suited for increased mass transfer from the soil to the water. Ten grams of Parrotfeather eliminated 35.8 mg of TNT from the soil within the first week, an 82% reduction. As such, any process that expedites the diffusion of TNT into water and/or promotes increased soil-plant enzyme contact could improve process efficiency.

Experimental evidence was also obtained that the presence of Parrotfeather in a TNT contaminated soil and water system promotes bacterial growth in the water. The Parrotfeather also appears to have the ability to limit, inhibit, and/or eliminate the unidentified component(s) acutely toxic to several aquatic organisms.

Recommendations

Parrotfeather has evidenced several attractive features that increase its potential for remediation of TNT contamination. Next, a full scale field demonstration must be designed and implemented. Implementation, however, must

be contingent upon incorporating into the final design a means to prevent the release of intermediate products.

Several areas were identified in this research that are recommended for further study. These could not be thoroughly investigated in this research effort due to limited resources and time.

- Photolytic Products and Toxicity:

Because any phytoremediation method requires sunlight, the photolytic transformation of TNT must be thoroughly investigated to determine the quantity, composition, and toxicity of the resulting products. Photolysis appears to attenuate the aqueous phase TNT concentrations and promote the formation of colored complexed materials as well as other colorless photo transformation products. These products could be responsible for the reduced microbial populations, aquatic organism deaths, and absence of algal growth observed in the field experiment controls.

- Parrotfeather Plants:

For phytoremediation with Parrotfeather to be a continuous process, additional studies must be performed to determine how to keep the plants alive and healthy. Further, the fate and toxicity of the ring-

opened components, likely incorporated into plant tissues, must be determined.

- Enhance TNT Mass Diffusion:

TNT mass diffusion from the soil into water is thought to be the rate limiting step. As such, any physical, chemical, or mechanical means of accelerating this diffusion process is an area worthy of additional research.

In the same vein, because an in situ process would likely have to deal with heterogeneous TNT contaminated soil containing TNT nuggets, the kinetics of nugget dissolution should be studied.

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APPENDIX A
TNT EXTRACTION PROCEDURE

OVERVIEW

Analysis for the TNT concentration of a soil requires an extraction procedure to remove the TNT from the contaminated soil medium. In the first field experiment, this consisted of adding enough solvent to a soil sample to dissolve the solid TNT material. As the field soil to be sampled in this experiment was supersaturated, each sample was dried at 60°C in a forage dryer. Once dry, a one gram subsample of the dried soil was crushed to a powder then placed in a screw top vial with 10 ml solvent. Each sample vial was secured in a test tube rack and mounted in a horizontal position on a beaker shaker. Once attached securely, samples were agitated for 24 hours at 3 revolutions per second. Overall, the TNT extraction procedure was: dry soil, select and measure a one gram subsample, crush subsample, then finally add solvent and agitate. After a short settling period, one ml TNT-solvent samples were taken for subsequent dilution and TNT concentration determination.

Problems were quickly observed with this TNT-soil extraction procedure. Random samples, set aside after routine analysis, were observed to undergo a slight aqueous color change which became more pronounced over time, from a light red to a much deeper red over a six to eight week

period. Another TNT concentration determination, on a random selection of these aged samples, exhibited a TNT concentration increase from the initial determinations. Significantly, the increase was about 22% above the previous determinations.

This shows all of the TNT was not dissolved into the solvent solution with that extraction procedure. Therefore, a series of laboratory experiments were conducted to check the relative error of different procedures and determine the best method for these experiments. Two different methods were compared, single and multiple extraction. Also, in the event that increased solvent volume would ensure adequate extraction with the single extraction method, the impact of two different solvent volumes was investigated.

Extraction Experiment Methods

A grab sample of TNT contaminated soil was removed from the prepared soil stockpile used in the first field experiment and dried at 60°C for 3 days. Two hundred grams of this dried soil was removed and crushed to a powder by hand with a mortar and pedestal to preclude the TNT nugget effect. After a thorough mixing, one gram subsamples were randomly selected as experimental samples.

A. Single Extraction:

Forty samples of dry powdered soil, one gram each, were placed in screw top vials. Solvent was added; 25 samples received ten ml and 15 samples received 20 ml. All 40 samples were mounted, in a horizontal position, onto the beaker shaker and agitated at three cycles per second for 24 hours.

After a one hour settling period, ten samples of both 10 ml and 20 ml solvent compositions were randomly selected and analyzed. Data from this comparison would be used to determine if a larger solvent volume resulted in a significant TNT concentration increase in solution. The remaining twenty samples were saved for later analysis to determine if additional TNT would be extracted from the soil and move into solution. If the TNT concentration did show an increase after setting for some time, then the single extraction procedure, as employed here and with this soil, would not prove adequate for TNT soil extraction.

On day 26, after the original forty samples were agitated, six of the remaining 10 ml solvent samples were randomly selected and analyzed. Another six of the remaining 10 ml solvent samples were also randomly selected and analyzed on day 56. As the observed color change and corresponding sample TNT concentration increase occurred

over a six to eight week timeframe, these aged samples were selected to cover that approximate timeframe.

B. Multiple Extraction:

Six samples, one gram each, of the prepared dry powdered soil used in the single extraction experiment was subjected to three separate solvent extractions. The first extraction consisted of adding 10 ml of solvent to the dry soil, securing the samples in the shaker, in a horizontal position, and agitating at 5 revolutions per second for 30 minutes. Then, the TNT-solvent solution was removed and another 10 ml of solvent was added to each soil sample. The samples were again shaken for 30 minutes. The process was repeated for a total of 3 extractions. All three, 30 ml total, TNT-solvent solutions from each sample were combined and vortex mixed for minute to provide the final sample for TNT concentration determination.

Results

All TNT concentrations were determined by reverse phase HPLC analysis. Also, samples were diluted by standard dilution methods to obtain a sample with the TNT concentration falling within the linear detection range of the HPLC.

A) Single Extraction:1) Solvent Volume

Ten samples of the 10 ml and another ten of the 20 ml solvent volumes were analyzed for TNT concentration. This TNT concentration data for each solvent volume series is listed in Table A1. An average TNT concentration of 4510 ppm and 4620 ppm was determined for the 10 and 20 ml samples respectively.

2) Time-series

Six of the remaining original 10 ml solvent samples were randomly selected for TNT concentration determinations on day 26 and another six were selected on day 56. A listing of these time series TNT concentration data are shown in Table A2. Their average TNT concentration, in ppm, was 5259 and 5413 for the day 26 and day 56 respectively.

B) Multiple Extraction:

Six samples were subjected to three separate extractions by 10 ml of solvent. At the conclusion of the third extract agitation cycle, the 30 ml of TNT-solution removed from each sample was vortex mixed for one minute. From this composite solution composed of all three TNT-

solvent solutions, a subsample was removed and analyzed for the TNT concentration.

Analysis of the single TNT-solvent solution at the end of the third agitation cycle showed the average concentration in that step was only about 80 ppm. To ensure full TNT removal from the soil sample, a forth extraction was performed and its TNT-solution was also analyzed for TNT, however, the concentration was well below one ppm indicating less than 10 ppm of solvent extractable TNT remained in the soil after three extractions. Therefore, the forth step was not included in this data and is not needed.

TNT concentration data resulting from the summation of the three extractions is listed in Table A3. This method resulted in an average TNT concentration of 5848 ppm.

ANALYSIS

A t-test for two samples was used to determine significant differences between the mean TNT concentrations at the 5% level of significance. The Quattro Pro for Windows Version 5 program was the statistical program used for this determination.

1) Solvent Volume

There was no significant difference between the means of the single extraction solvent volumes, using 10 ml and 20 ml of solvent, at the 5% level of significance. Assuming the greater mean to be the true mean, using a single 10 ml solvent extraction yields a 2% relative error. Based on this data, increasing the solvent volume to 20 ml would not change the results by any significant amount.

2) Time-series

There is a significant difference between the means of this series and the original 10 ml sample series at the 5% significance level. Between the initial series and the day 26 series, there is a 14% relative error, again assuming the greater TNT concentration mean to be the more correct value.

This shows that the TNT concentration does increase in solution. Thus, the single extraction method using 10 ml of solvent and 24 hours of agitation results in a large error when working with this TNT contaminated soil.

3) Multiple Extraction

There is a significant difference between the multiple extraction mean and the initial 10 ml single extraction mean at the 5% significance level. If the higher TNT concentration is accepted as the more correct value, there is a 23% relative error between methods.

Additionally, there is a 10% relative error between this multiple extraction series and the single extraction day 56 time series. Therefore, the multiple extraction method used in this experiment results in a better extraction of TNT from this TNT contaminated soil.

Conclusion

Multiple extractions must be used to extract the TNT from this contaminated soil. Likely, the TNT concentration may be greater than can be solubilized in only 10 ml of solvent with the prescribed agitation scheme.

Additional experiments were performed to investigate other aspects of the extraction process such as solvent strength and mixing times. From this, it was determined that the best extraction procedure parameters for this TNT contaminated soil are: solvent strength - 100% acetonitrile,

number of extractions - three, sample shaking time at five rpms - 30 minutes per extraction, and solvent volume - 10 ml.

Table A1

Extraction Procedure TNT Concentration Comparison

10 ml Samples TNT (ppm)	20 ml Samples TNT (ppm)
5132	4329
4935	4676
4347	4317
3968	4891
4245	4459
4890	4951
4642	4685
4244	4420
4344	4856
4357	4612

Table A2

Aged Sample TNT Concentration Comparison

Day 26 TNT (ppm)	Day 56 TNT (ppm)
5253	5696
5278	5164
5208	5511
5259	5436
5119	5490
5438	5182

Table A3

Multiple Extraction Procedure TNT Concentration

TNT (ppm)
5944
5911
5858
5658
6043
5672

APPENDIX B
FIELD EXPERIMENT ONE DATA

Table B1

Field Experiment One: TNT Concentration in the Soil of the Field Containers

(Page 1 of 6)

Pool	Sample Location	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
		TNT (ppm)	TNT (ppm)	TNT (ppm)	TNT (ppm)	TNT (ppm)	TNT (ppm)
1	NW	9878	3321	2318	8431	2686	3894
	NE	5697	5145	4147	5359	3294	3956
	SW	4684	3949	3429	3328	4060	3432
	SE	4864	1999	2771	2904	5609	3984
2	NW	2669	3851	3030	3873	4897	3832
	NE	8964	3295	3472	5426	3426	3650
	SW	4640	4049	3168	2461	4314	5035
	SE	4115	7295	1911	3567	3138	4010
3	NW	5448	1273	4082	5583	5347	5129
	NE	9334	3868	3750	5127	12643	8977
	SW	5965	6329	2612	4515	4477	4035
	SE	5618	3159	4041	3339	2768	4834

Note: Shaded values result from the treatment with no Parrotfeather.

Table B1

Field Experiment One: TNT Concentration in the Soil of the Field Containers

(Page 2 of 6)

		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
	Sample	TNT	TNT	TNT	TNT	TNT	TNT
Pool	Location	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
4	NW	8893	8459	12038	4057	3184	4097
	NE	32536	6482	4268	3507	3651	8886
	SW	5650	5655	3607	4254	4015	3185
	SE	8402	2813	3087	4405	4488	4606
5	NW	6298	12026	3494	14525	2822	4468
	NE	6394	18245	3902	5997	3579	7285
	SW	7199	5279	6943	7233	2075	48840
	SE	9135	4203	13417	2165	4519	4148
6	NW	4185	13129	7071	3337	1677	10507
	NE	3089	8818	6991	5363	7754	6828
	SW	4627	3630	2918	3666	7283	9340
	SE	6978	5400	9455	2566	2529	6019

Note: Shaded values result from the treatment with no Parrotfeather.

Table B1

Field Experiment One: TNT Concentration in the Soil of the Field Containers

(Page 3 of 6)

		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
	Sample	TNT	TNT	TNT	TNT	TNT	TNT
	Location	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
7	NW	4535	4465	16230	6334	4089	4477
	NE	4784	3768	9418	3000	17577	14186
	SW	2636	3979	2490	3341	2711	3088
	SE	5438	3145	6619	3187	3486	5809
8	NW	3779	3620	3271	3968	7161	2485
	NE	18309	4708	3527	5774	3834	10097
	SW	8688	2538	3937	3313	3197	3353
	SE	11793	5416	2587	2803	2914	5519

Table B1

Field Experiment One: TNT Concentration in the Soil of the Field Containers

(Page 4 of 6)

		Week 8	Week 9	Week 10	Week 11	Week 13
	Sample	TNT	TNT	TNT	TNT	TNT
Pool	Location	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
1	NW	2679	6928	7967	3173	4103
	NE	4788	2575	6503	2975	2400
	SW	3513	2581	2665	2614	3064
	SE	3096	3026	2398	7972	4592
2	NW	4213	2806	2347	4532	2813
	NE	3453	2547	3165	2897	3078
	SW	10265	3162	2777	2316	2263
	SE	3153	3173	3075	3152	19850
3	NW	4055	6826	8248	1908	3047
	NE	4335	4036	7730	2661	3893
	SW	7586	3800	3210	2846	8868
	SE	2839	5532	3240	2746	3349

Note: Shaded values result from the treatment with no Parrotfeather.

Table B1

Field Experiment One: TNT Concentration in the Soil of the Field Containers

(Page 5 of 6)

		Week 8	Week 9	Week 10	Week 11	Week 13
	Sample	TNT	TNT	TNT	TNT	TNT
Pool	Location	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
4	NW	6977	3424	4697	3380	13498
	NE	4568	4169	3988	6382	3076
	SW	4150	5170	3264	6349	2838
	SE	12608	4174	2953	1939	5419
5	NW	4218	2171	2834	2441	1909
	NE	2725	3544	4937	3714	3973
	SW	6963	3581	1915	3235	3467
	SE	2967	3146	8101	2852	3486
6	NW	7082	4244	2056	3099	2999
	NE	5834	2863	3663	1575	4483
	SW	6409	7280	3999	2455	3522
	SE	4809	3192	3924	4757	3731

Note: Shaded values result from the treatment with no Parrotfeather.

Table B1
 Field Experiment One: TNT Concentration in the Soil of the Field Containers
 (Page 6 of 6)

		Week 8	Week 9	Week 10	Week 11	Week 13
	Sample	TNT	TNT	TNT	TNT	TNT
Pool	Location	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
7	NW	4419	2319	2790	3206	14678
	NE	4598	3099	5955	2283	2959
	SW	4055	2948	2045	3102	2231
	SE	14597	6018	3234	3743	3846
8	NW	2931	2513	1996	2797	3707
	NE	8060	3744	6988	2991	3618
	SW	4130	4770	2717	2086	2367
	SE	4942	3226	4472	3414	2290

Table B2

Field Experiment One: TNT Concentration in the Water of the Field Containers.

Pool	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 8
	TNT (ppm)	TNT (ppm)	TNT (ppm)	TNT (ppm)	TNT (ppm)	TNT (ppm)	TNT (ppm)
1	36	26	25	23	31	19	33
2	7	10	6	4	7	10	7
3	7	8	8	6	11	8	8
4	21	28	21	20	27	33	30
5	6	6	7	5	8	9	7
6	6	8	9	7	8	10	10
7	3	5	5	4	6	8	6
8	4	4	5	4	6	7	6

Note: Without plants treatments are lightly shaded for emphasis.

Table B3

Field Experiment One: Estimation of Microbial Numbers in the Soil
of the Field Containers

(Page 1 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
1A	0	0	0	0	0
1B	0	20	40	0	0
1C	0	0	0	0	0
2A	0	0	0	0	0
2B	0	0	20	0	0
2C	0	0	20	0	0
3A	0	0	20	0	0
3B	0	0	0	0	0
3C	0	0	20	0	0
4A	0	0	0	0	0

Table B3

Field Experiment One: Estimation of Microbial Numbers in the
Soil of the Field Containers

(Page 2 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
4B	0	20	0	0	0
4C	0	0	0	0	0
5A	0	0	0	0	0
5B	0	0	0	0	0
5C	0	0	0	0	0
6A	0	0	0	0	0
6B	0	0	0	0	0
6C	0	0	0	0	0
7A	0	0	0	0	0
7B	0	0	0	0	0
7C	0	0	0	0	0
8A	0	0	0	0	0
8B	0	0	0	0	0
8C	0	0	0	0	0

Table B4

Field Experiment One: Estimation of Microbial Numbers in
Water of the Field Containers

(Page 1 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
1A	40	100	20	0	0
1B	40	60	20	0	20
1C	40	20	0	0	60
2A	40	0	100	80	80
2B	40	40	60	0	0
2C	20	80	40	20	0
3A	80	80	20	40	20
3B	20	40	20	20	0
3C	60	0	60	40	20
4A	40	20	40	80	40

Table B4

Field Experiment One: Estimation of Microbial Numbers in
Water of the Field Containers

(Page 2 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
4B	20	40	20	40	40
4C	0	0	20	40	40
5A	100	120	180	160	120
5B	80	0	20	80	60
5C	0	40	20	60	20
6A	100	80	20	80	10
6B	40	20	40	0	20
6C	40	60	40	40	0
7A	20	40	20	40	20
7B	80	0	40	60	20
7C	40	80	20	80	0
8A	60	40	20	20	20
8B	80	0	0	40	40
8C	20	60	0	40	20

The average number of organisms found in the "with plants" treatment was 221 and in the "without plants" treatment was 150.

APPENDIX C
FIELD EXPERIMENT TWO DATA

Table C1

Field Experiment Two: Aqueous Phase TNT Concentration in the Soil Solution Containers

	Week	Week	Week	Week	Week	Week	Week
Pool	# 1	# 2	# 3	# 4	# 5	# 7	#15
1	0.2	0.3	ND	0.3	ND	0.1	ND
2	1.0	ND	8.0	7.0	11.6	7.3	16.1
3	11.1	10.8	8.0	9.0	11.2	6.0	16.2
4	0.1	0.2	0.1	1.8	ND	0.2	ND
5	0.6	1.0	0.2	0.1	ND	ND	ND
6	11.6	6.9	8.0	11.0	9.4	21.0	19.0
7	12.6	13.1	10.5	10.0	10.8	14.8	15.8
8	0.3	0.0	0.0	ND	ND	ND	ND
9	1.0	0.0	0.0	0.0	ND	ND	ND
10	1.0	4.0	9.0	8.6	15.6	15.3	19.1

Note: Without plant treatments are lightly shaded for emphasis.

Table C2

Field Experiment Two: Soil Redox Potential in the Solution Containers

(Page 1 of 3)

Soil Solution Containers										
Week 4	1	2	3	4	5	6	7	8	9	10
Pool										
N	73	545	576	25	280	375	121	490	380	424
S	404	450	471	57	396	326	191	462	395	477
C	218	440	438	415	11	160	452	384	325	389
E	392	538	543	-16	330	355	331	476	160	114
W	474	318	270	283	125	369	200	484	494	492
Avg	312	458	460	153	228	317	259	459	351	379

- Note 1) All values have been adjusted to the Hydrogen Reference Electrode Standard.
 Note 2) Shaded columns show readings from pools having the no Parrotfeather treatment.
 Note 3) Electrode location within each pool is identified by: N=North, S=South, C=Center, E=East, and W=West.

Table C2
 Field Experiment Two: Soil Redox Potential in the Solution Containers
 (Page 2 of 3)

Week 5	Soil Solution Containers									
Pool	1	2	3	4	5	6	7	8	9	10
N	460	405	318	87	257	345	100	233	204	423
S	348	342	525	290	178	546	246	404	214	352
C	132	261	428	197	95	145	231	261	14	218
E	306	342	529	90	183	218	224	459	276	125
W	14	480	519	217	353	215	230	441	251	267
Avg	252	366	464	176	213	230	206	360	192	277

Note 1) All values have been adjusted to the Hydrogen Reference Electrode Standard.
 Note 2) Shaded columns show readings from pools having the no Parrotfeather treatment.
 Note 3) Electrode location within each pool is identified by: N=North, S=South, C=Center, E=East, and W=West.

Table C2
 Field Experiment Two: Soil Redox Potential in the Solution Containers
 (Page 3 of 3)

Week 7	Soil Solution Containers									
Pool	1	2	3	4	5	6	7	8	9	10
N	330	214	247	21	206					
S	219	217	352	76	152					
C	58	202	254	68	2					
E	259	241	389	194	119					
W	-13	241	421	111	203					
Avg	171	223	333	94	136					

- Note 1) All values have been adjusted to the Hydrogen Reference Electrode Standard.
 Note 2) Shaded columns show readings from pools having the no Parrotfeather treatment.
 Note 3) Electrode location within each pool is identified by: N=North, S=South, C=Center, E=East, and W=West.
 Note 4) Incomplete data resulted from faulty meter.

Table C3
Field Experiment Two: Aqueous Phase pH Data

Time (Week)	TNT Contaminated Soil-Water Solution Containers									
	1	2	3	4	5	6	7	8	9	10
1	6.5	7.1	7.1	6.5	6.6	7.1	7.1	6.6	6.6	7.0
2	6.5	7.0	7.0	6.6	6.8	7.0	7.0	6.5	6.6	7.0
4	6.6	7.2	7.1	6.6	6.8	7.2	7.1	6.8	6.8	7.1

Note: Without plants treatments are lightly shaded for emphasis.

Table C4

Field Experiment Two: Estimation of Microbial Numbers in
the Soil of the Field Containers

(Page 1 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
1A	0	0	0	0	0
1B	0	0	0	0	0
1C	0	0	0	0	0
2A	0	0	0	0	0
2B	0	0	0	0	0
2C	0	0	0	0	0
3A	0	0	0	0	0
3B	0	0	0	0	0
3C	0	0	0	0	0
4A	0	0	0	0	0
4B	0	0	0	0	0
4C	0	0	0	0	0
5A	0	0	0	0	0
5B	0	0	0	0	0

Table C4

Field Experiment Two: Estimation of Microbial Numbers in
the Soil of the Field Containers

(Page 2 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
5C	0	0	0	0	0
6A	0	0	0	0	0
6B	0	0	0	0	0
6C	0	0	0	0	0
7A	0	0	0	0	0
7B	0	0	0	0	0
7C	0	0	0	0	0
8A	0	0	0	0	0
8B	0	0	0	0	0
8C	0	0	0	0	0
9A	0	0	0	0	0
9B	0	0	0	0	0
9C	0	0	0	0	0
10A	0	0	0	0	0
10B	0	0	0	0	0
10C	0	0	0	0	0
Soil 1	0	0	0	0	0
Soil 2	0	0	0	0	0
Soil 3	0	0	0	0	0

Table C5

Field Experiment Two: Estimation of Microbial Numbers in
the Water of the Field Containers

(Page 1 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
1A	0	0	20	0	0
1B	20	100	40	0	40
1C	40	140	60	100	40
2A	20	0	60	0	20
2B	0	20	0	20	40
2C	0	0	0	20	0
3A	0	20	40	0	20
3B	20	40	40	20	40
3C	20	0	0	0	20
4A	20	80	0	60	0
4B	120	20	80	120	80
4C	100	20	60	0	0
5A	20	20	60	20	20
5B	40	40	20	40	20

Table C5

Field Experiment Two: Estimation of Microbial Numbers in
the Water of the Field Containers

(Page 2 of 2)

Active Bacteria Units per Field					
Sample	1	2	3	4	5
5C	20	0	0	60	100
6A	0	0	0	20	20
6B	0	0	40	40	40
6C	0	0	0	0	0
7A	0	0	20	0	40
7B	140	0	0	0	0
7C	0	0	0	0	0
8A	0	60	20	0	60
8B	80	60	40	40	20
8C	40	40	60	40	40
9A	20	40	0	0	0
9B	20	80	100	40	60
9C	100	40	40	20	40
10A	20	0	0	0	20
10B	0	0	0	0	0
10C	40	60	20	0	0
Pond 1	40	20	40	80	60
Pond 2	60	40	20	40	40
Pond 3	80	80	60	80	40

APPENDIX D
LAB EXPERIMENTS

Table D1
Parrotfeather Effect on the Aqueous Phase TNT Concentration
(Page 1 of 2)

Time (Hour)	TNT Concentration (ppm)									
	Beaker									
	1	2	3	4	5	6	7	8	9	10
0.5	88.7	64.4	64.7	64.3	90.3	86.2	66.4	61.4	81.7	76.3
1.0	83.8	60.2	59.0	67.7	87.0	81.0	63.5	62.8	89.2	84.8
1.5	85.8	58.9	50.1	61.7	84.5	84.0	57.2	54.7	81.3	79.0
2.0	86.4	44.3	49.1	57.4	82.6	77.5	61.3	58.4	85.3	84.7
2.5	79.3	56.7	60.8	63.0	85.2	74.0	30.0	40.0	76.0	74.0
3.0	84.9	44.7	32.8	43.3	83.6	82.9	57.4	51.4	77.1	84.7
3.5	81.5	47.5	46.2	49.7	86.5	78.1	50.9	50.1	86.6	82.4
4.0	84.9	43.5	48.0	48.9	79.5	78.9	45.1	46.8	76.0	76.7
5.0	81.6	44.2	57.1	56.2	84.5	85.7	55.4	51.3	83.8	84.2
6.0	84.1	39.3	54.8	56.1	88.4	83.0	53.7	52.8	83.5	91.3
7.0	94.3	48.1	56.4	57.0	86.8	89.3	53.9	55.7	93.0	89.2

Note1: Without plants treatments are lightly shaded for emphasis.

Note2: TNT concentrations of ND = None Detected.

Table D1
Parrotfeather Effect on the Aqueous Phase TNT Concentration
(Page 2 of 2)

Time (Hour)	TNT Concentration (ppm)									
	Beaker									
	1	2	3	4	5	6	7	8	9	10
8	87.8	39.0	51.5	50.6	91.7	90.8	52.9	54.4	92.8	85.5
15	85.0	32.9	48.0	44.1	85.4	90.5	47.0	45.6	91.8	62.7
26	93.0	19.6	41.5	35.8	92.0	87.4	40.2	38.4	87.0	87.7
30		14.9	37.3	28.2			36.6	35.0		
37		9.3	30.0	20.5			28.1	25.2		
50	84.8	0.0	20.5	6.7	83.5	83.1	12.8	9.2	89.8	87.0
61		0.0	13.2	3.4			10.1	3.8		
77	96.2	ND	1.1	ND	98.0	99.3	2.0	ND	97.5	97.8
101	79.7	0.3	0.4	0.2	82.1	82.9	0.1	ND	80.9	86.1
147	51.0	0.6	0.6	ND	86.6	85.3	0.7	ND	83.4	90.8
30 Days	83.0	ND	ND	ND	91.0	90.0	ND	ND	90.0	93.0

Note1: Without plants treatments are lightly shaded for emphasis.

Note2: TNT concentrations of ND = None Detected.

Table D2
Parrotfeather Effect on the Solid Phase TNT Concentration

TNT Concentration (ppm)										
Beaker										
Step	1	2	3	4	5	6	7	8	9	10
1	169.2	158.2	162.9	149.5	165.4	147.9	164.6	151.4	165.9	187. 3
2	0.0	158.4	151.2	141.4	0.0	152.6	1.0	136.4	4.0	1.0
2	22.3	144.4	145.7	139.7	43.6	133.8	47.1	152.3	37.1	59.4
3	2.3	144.5	147.6	144.3	60.6	139.8	0.0	163.6	0.0	5.0
3	0.0	153.7	162.1	168.0	93.0	190.9	67.6	183.5	37.2	32.5
4	0.0	172.8	162.4	173.0	0.0	197.4	0.0	193.6	0.0	0.0
5	ND	150.2	143.5	139.4	0.1	138.9	2.0	168.0	0.2	5.2

Note1: Without plants treatments are lightly shaded for emphasis.

Note2: TNT concentrations of ND = None Detected.

Table D3
 Ten Week Study - TNT Concentration in Water of "With Plants" Treatment
 (Page 1 of 2)

With Plants Treatment - TNT Concentration (ppm)												
Week	Beaker 1			Beaker 2			Beaker 3					
1	0.2	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	ND	ND	0.0	ND	0.0	ND	0.0	ND	ND	ND
3	0.5	0.2	0.3	1.3	0.8	ND	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	0.0	ND	ND	0.0	ND	0.0	0.0	0.0	0.0	ND	ND	ND
6	0.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	ND	ND	ND	0.0	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Note: TNT concentrations of ND = None Detected.

Table D3
 Ten Week Study - TNT Concentration in Water of "Without Plants" Treatment
 (Page 2 of 2)

Without Plants Treatment - TNT Concentration (ppm)											
Week	Beaker 1			Beaker 2			Beaker 3				
1	153.5	150.3	149.8		167.4	164.1	151.7		151.8	141.1	145.4
2	147.8	153.2	152.5		153.2	161.5	160.9		159.1	149.1	168.4
3	96.3	120.5	114.1		120.7	130.2	115.6		103.9	129.6	117.5
4	109.1	124.5	124.6		93.2	105.3	137.2		133.3	102.4	130.2
5	133.9	136.0	105.7		133.3	133.2	112.6		129.8	133.3	128.4
6	100.6	101.7	105.3		122.6	118.8	122.5		98.6	107.9	119.8
7	104.3	107.0	124.2		122.4	130.7	109.5		108.4	112.9	124.3
8	84.5	95.5	89.1		85.3	88.5	97.4		80.6	71.6	80.0
10	103.7	93.0	98.3		98.6	79.9	79.0		80.7	89.9	90.2

Table D4
Ten Week Study - TNT Concentration Remaining in the Contaminated Soil

Week	Treatments					
	With Plants			Without Plants		
	Beaker 1 (ppm)	Beaker 2 (ppm)	Beaker 3 (ppm)	Beaker 1 (ppm)	Beaker 2 (ppm)	Beaker 3 (ppm)
1	862	817	681	4154	2247	2621
2	558	483	851	2592	2656	2811
3	248	157	235	2818	2336	2872
4	250	179	235	2578	2588	2382
5	292	234	182	3025	3121	2388
6	151	209	158	3172	2925	2152
7	141	138	266	2656	2434	2390
8	305	67	97	2608	2352	2636
10	86	151	154	2966	3148	2595

Note1: Without plants treatments are lightly shaded for emphasis.

Note2: The Initial Average TNT Soil Concentration = 4,376 ppm.

Table D5
Percentage of ^{14}C Activity Recovered for Each Experimental Component

Percentage of ¹⁴ C Activity Recovered For Each Experimental Component							
Time (Hours)	Plant Wash (%)	Mortar Wash (%)	Aqueous Solution (%)	Plant Extraction (%)	Plant Oxidation (%)	CO ₂ (%)	Total (%)
3	24	0	40	31	11		106
12	24	0	35	27	15		101
24	16	0.5	33	31	22		103
48	10	0.2	38	30	25		103
73	11	0	28	24	25	< 1 %	88

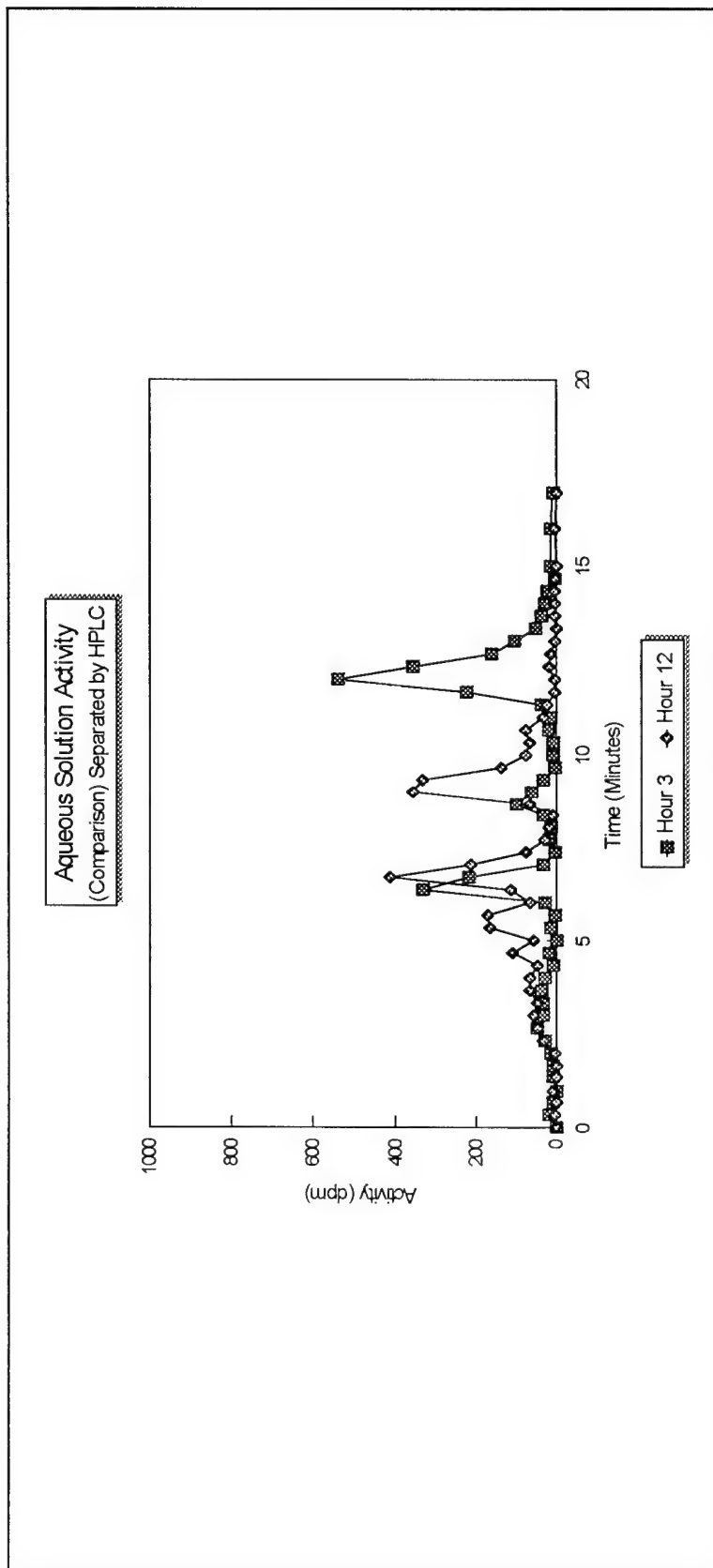


Figure D1
Comparison Plot of Aqueous Solution Activity, Hours 3 and 12.

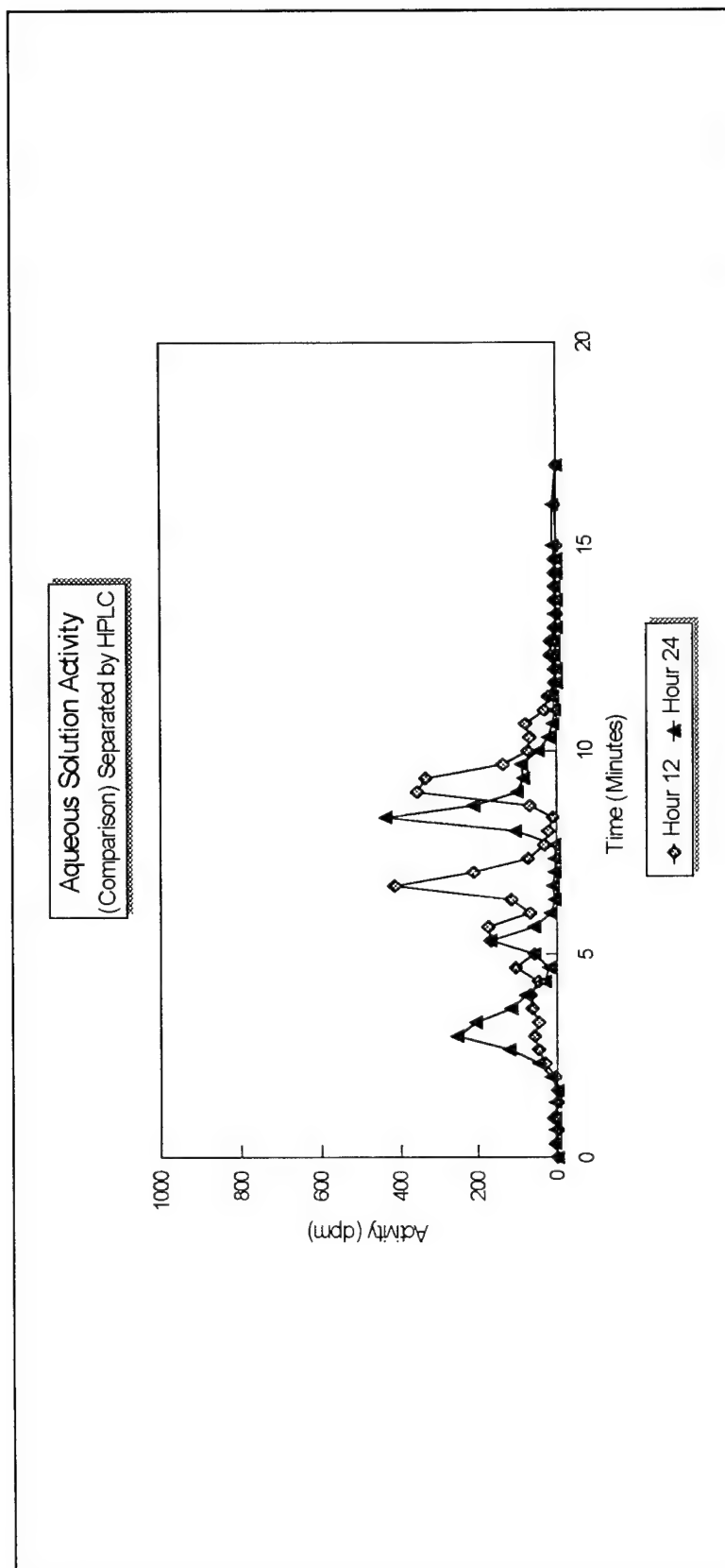


Figure D2
Comparison Plot of Aqueous Solution Activity, Hours 12 and 24.

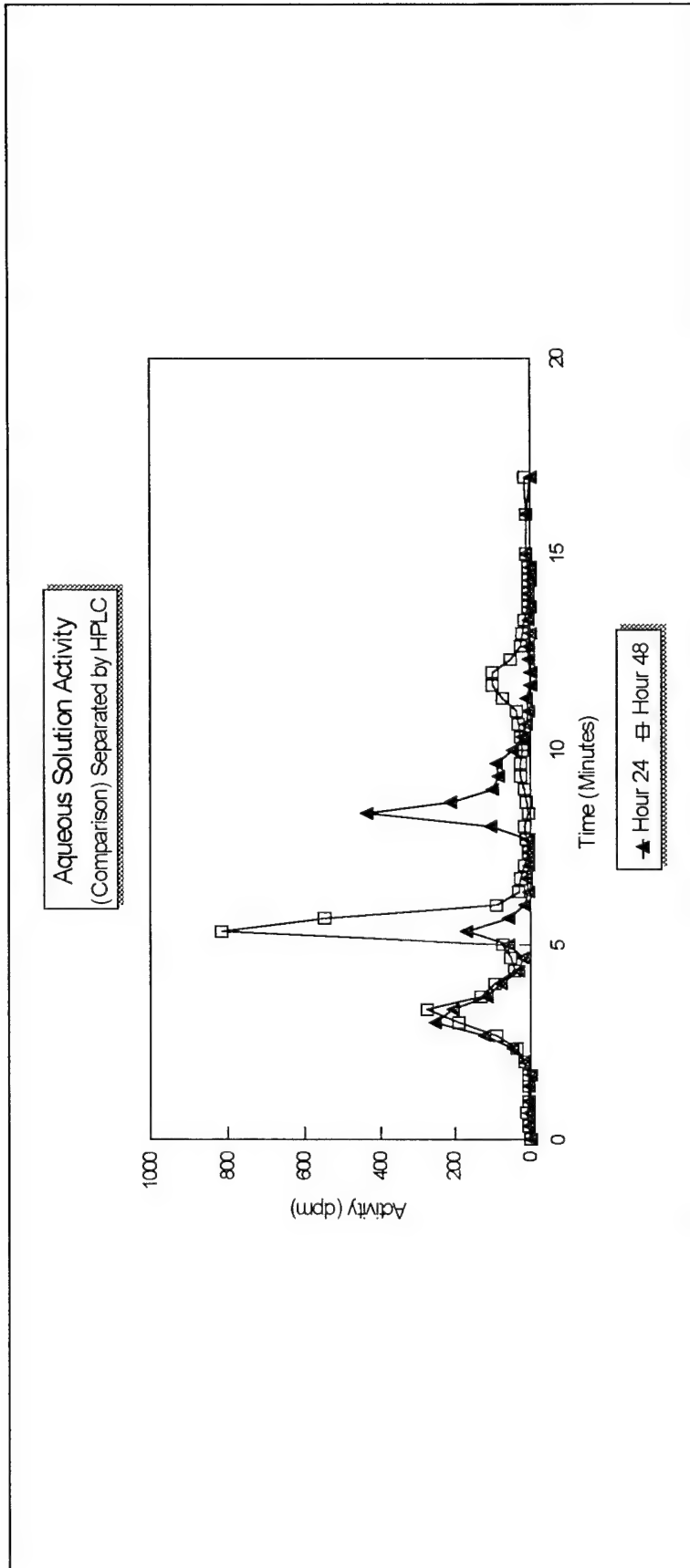


Figure D3
Comparison Plot of Aqueous Solution Activity, Hours 24 and 48.

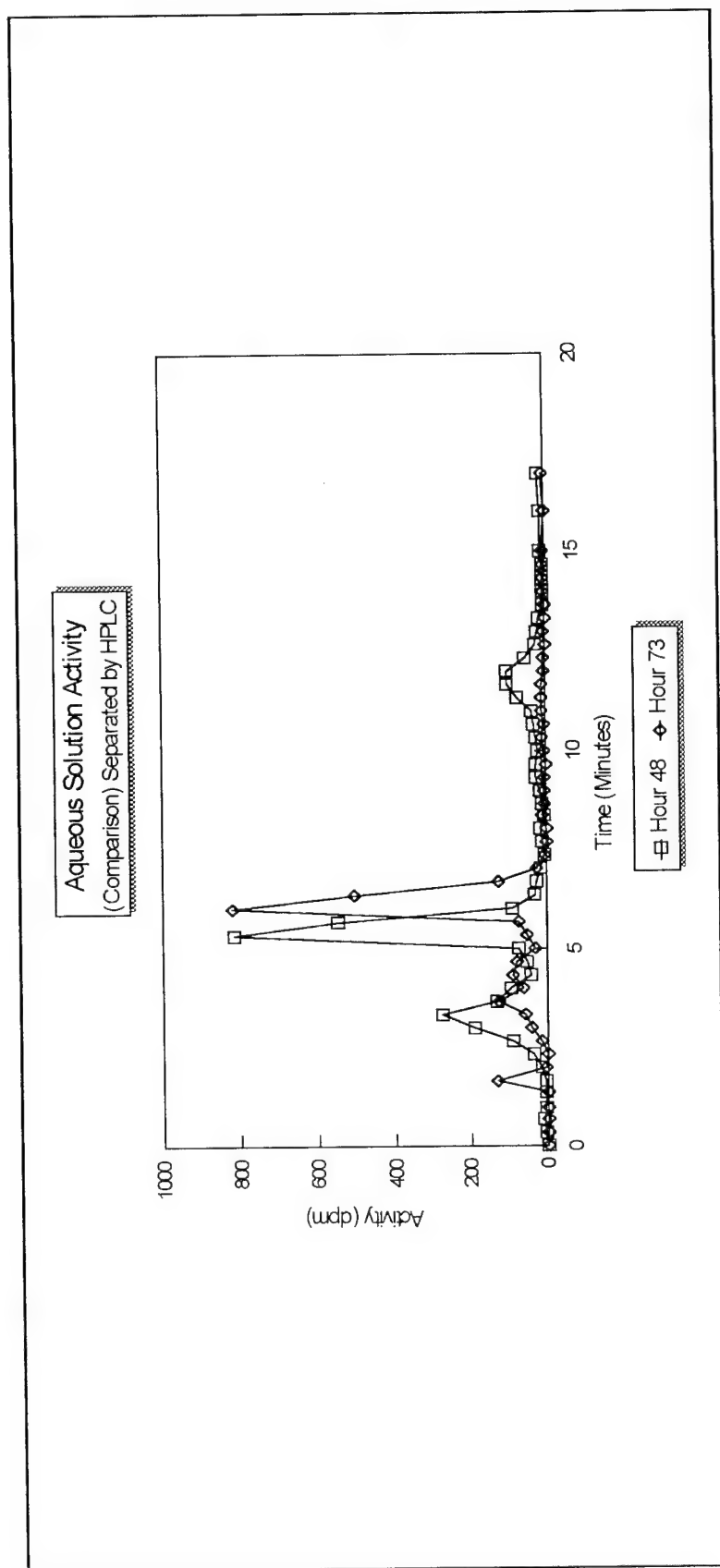


Figure D4
Comparison Plot of Aqueous Solution Activity, Hours 48 and 73.

Table D6
 Aqueous Phase ^{14}C Activity After Separation by HPLC
 Page 1 of 4

Time	^{14}C Aqueous Phase Activity (dpm)				
(min)	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
0.0	0	0	0	0	0
0.3	20.74	5.77	3.75	7.48	2.24
0.7	11.08	0	7.55	10.2	1.36
1.0	0.97	11.47	6.24	4.89	0
1.3	12.4	0.52	4.02	7.53	3.53
1.7	10.62	0	0	7.53	136
2.0	16.31	7.98	17.55	15.42	4.44
2.3	28.16	33.06	48.4	40.02	1.35
2.7	47.01	48.89	120.83	90.18	14.96
3.0	35.18	57.65	257.5	189.57	44.87
3.3	35.13	47.56	206.53	273.99	56.73
3.7	38.69	66.89	117.76	132	129.84
4.0	28.17	68.66	82.23	96.27	65.02
4.3	8.85	50.16	34.78	45.33	89.2
4.7	18.52	108.17	21.16	55.41	79.27
5.0	1.85	56.85	59.36	74.24	33.86
5.3	14.13	168.18	168.15	813.47	51.94
5.7	6.23	173.43	57.62	544.76	75.59

Table D6
Aqueous Phase ^{14}C Activity After Separation by HPLC
Page 2 of 4

Time	^{14}C Aqueous Phase Activity (dpm)				
(min)	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
6.0	28.17	68.99	16.34	92.68	819.27
6.3	330.8	116.07	5.78	32.56	505.92
6.7	217.25	413.87	11.07	28.15	129.19
7.0	32.11	212.43	3.58	18.03	25.52
7.3	4.49	76.94	8.85	7.51	4.47
7.7	18.94	30.36	7.11	11.91	0.94
8.0	15.89	21.99	108.2	17.6	2.28
8.3	32.59	10.15	434.11	7.91	9.28
8.7	100.18	69.08	215.26	13.58	6.64
9.0	61.08	354.8	100.63	16.63	5.31
9.3	32.1	333.89	83.98	28.05	4.45
9.7	4.04	136.9	90.05	29.83	2.66
10.0	11.07	75.68	50.59	23.21	5.76
10.3	12.38	68.17	19.85	28.48	11.5
10.7	19.41	79.22	12.8	35.09	5.77
11.0	14.58	32.15	4.08	39.01	11.44
11.3	38.25	22.9	9.76	72.43	11.46
11.7	222.63	7.08	0.08	101.8	11.47
12.7	162.65	17.26	5.78	28.96	3.12
13.0	104.78	7.97	0	24.57	6.2

Table D6
 Aqueous Phase ^{14}C Activity After Separation by HPLC
 Page 3 of 4

Time (min)	^{14}C Aqueous Phase Activity (dpm)				
	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
12.0	541.54	7.98	0	100.48	3.99
12.3	357.93	18.56	4.51	54.45	7.96
13.3	51.35	0	4.03	18.89	3.14
13.7	39.09	4.48	0	5.29	2.7
14.0	27.72	5.83	8.42	7.5	4.45
14.3	22.86	7.96	2.71	7.05	8.83
14.7	7.53	4.5	3.16	5.29	5.36
15	14.56	0	10.18	9.69	7.5
16	17.07	8.27	9.58	13.45	0.26
17	11.79	2.09	0.74	14.78	5.99
18	21.91	6.5	2.5	13.04	3.36
19	5.65	3.84	0	15.24	10.42
20	6.07	1.63	8.27	16.13	7.35
21	11.37	8.28	6.49	23.65	5.99
22	6.5	15.81	6.03	9.06	4.68
23	11.37	3.39	2.96	15.24	5.12
24	8.73	2.5	8.21	5.53	8.66
25	14.41	1.63	5.57	9.51	13.12

Table D6
Aqueous Phase ^{14}C Activity After Separation by HPLC
Page 4 of 4

Time	^{14}C Aqueous Phase Activity (dpm)				
(min)	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
26	1.21	0.76	2.05	16.12	4.22
27	8.7	0	3.82	13.03	2.04
28	8.72	0	0	5.49	0
29	5.16	0.3	11.81	11.27	3.8
30	15.34	0	0	0	6.87

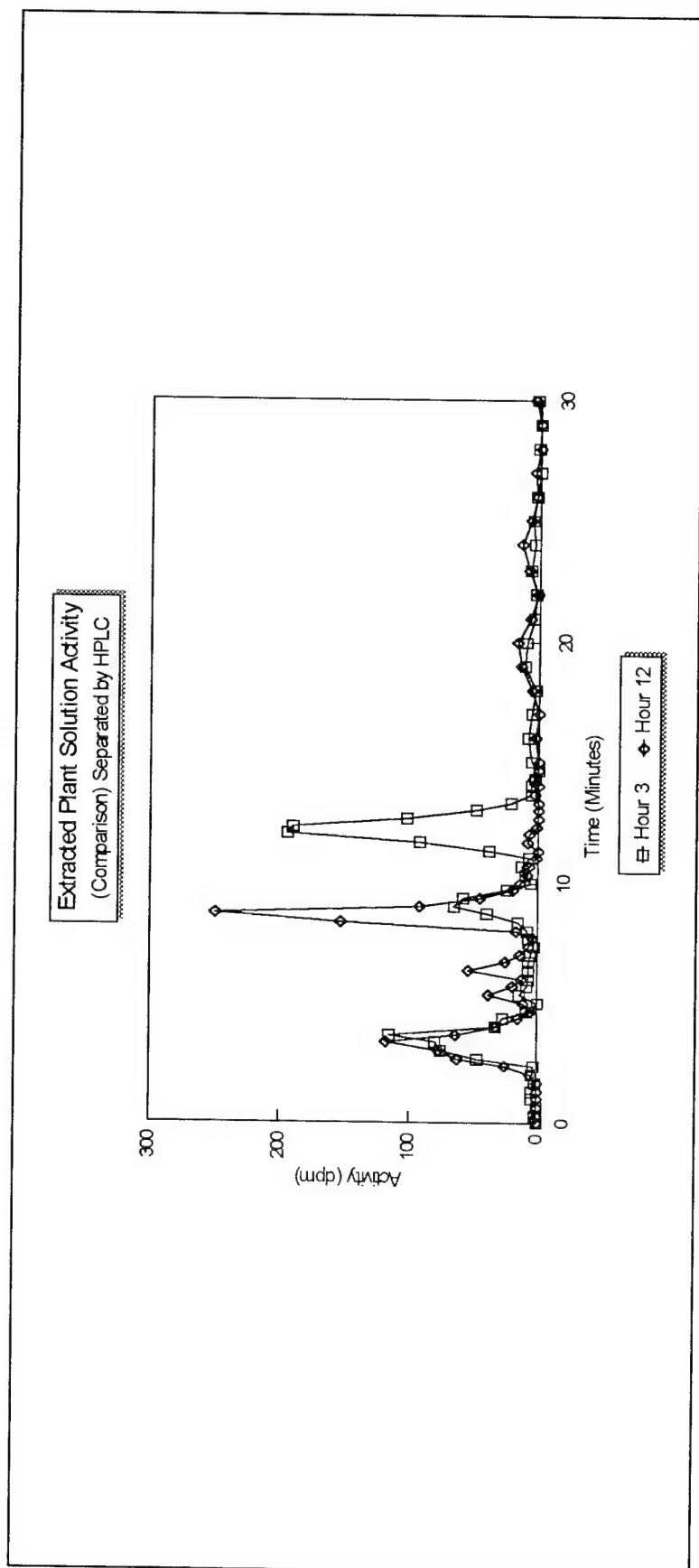


Figure D5
Extrated Plant Solution Activity Comparison, 3 and 12 hours.

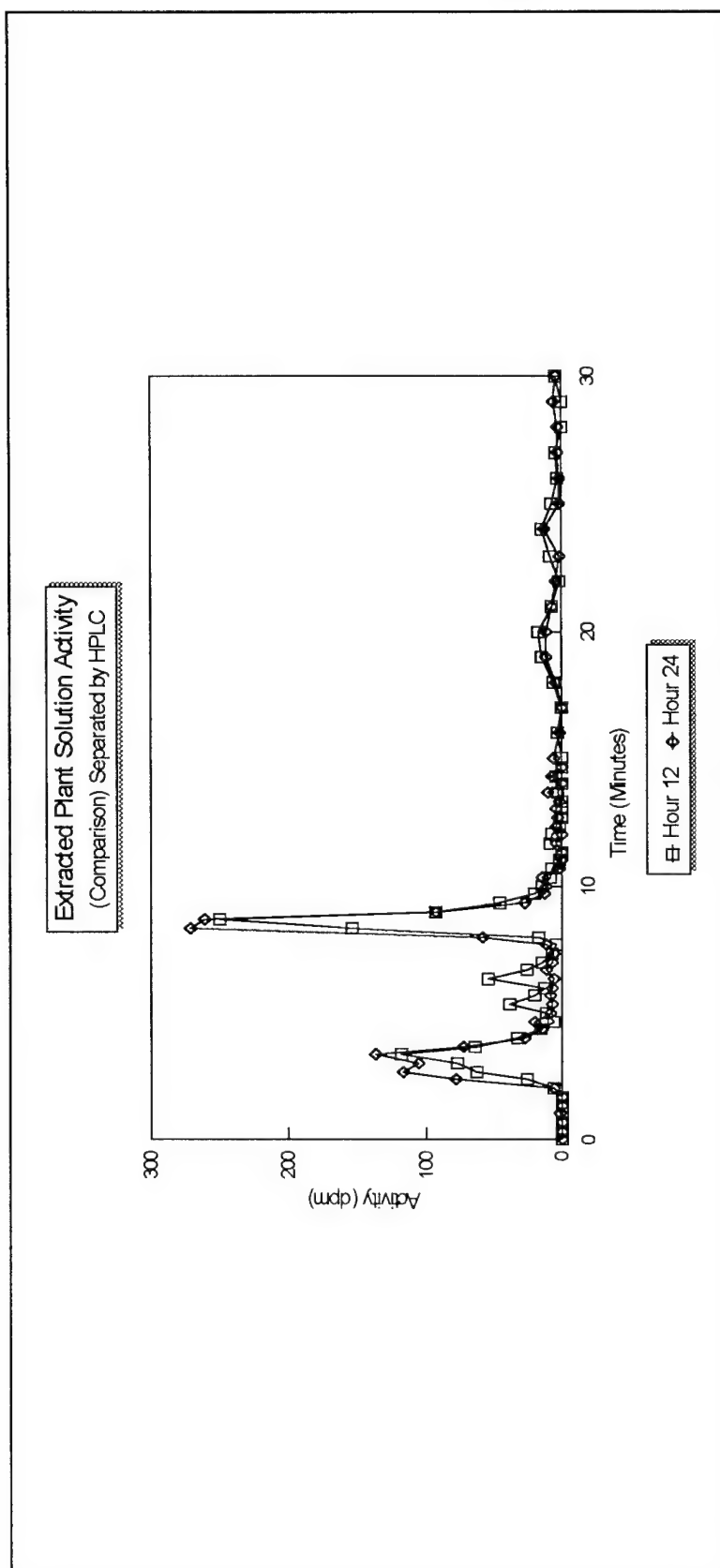


Figure D6
Extracted Plant Solution Activity Comparison, 12 and 24 hours.

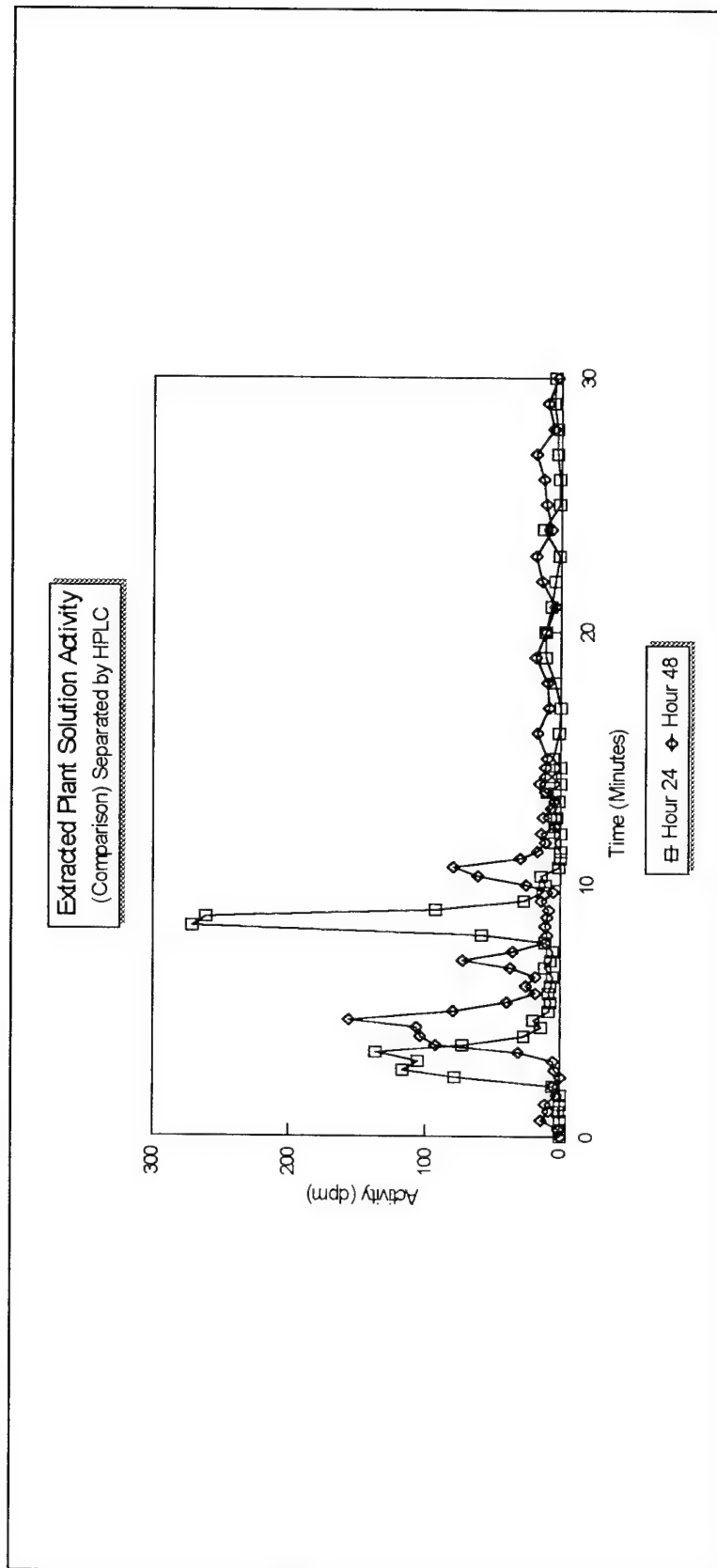


Figure D7
Extracted Plant Solution Activity Comparison, 24 and 48 hours.

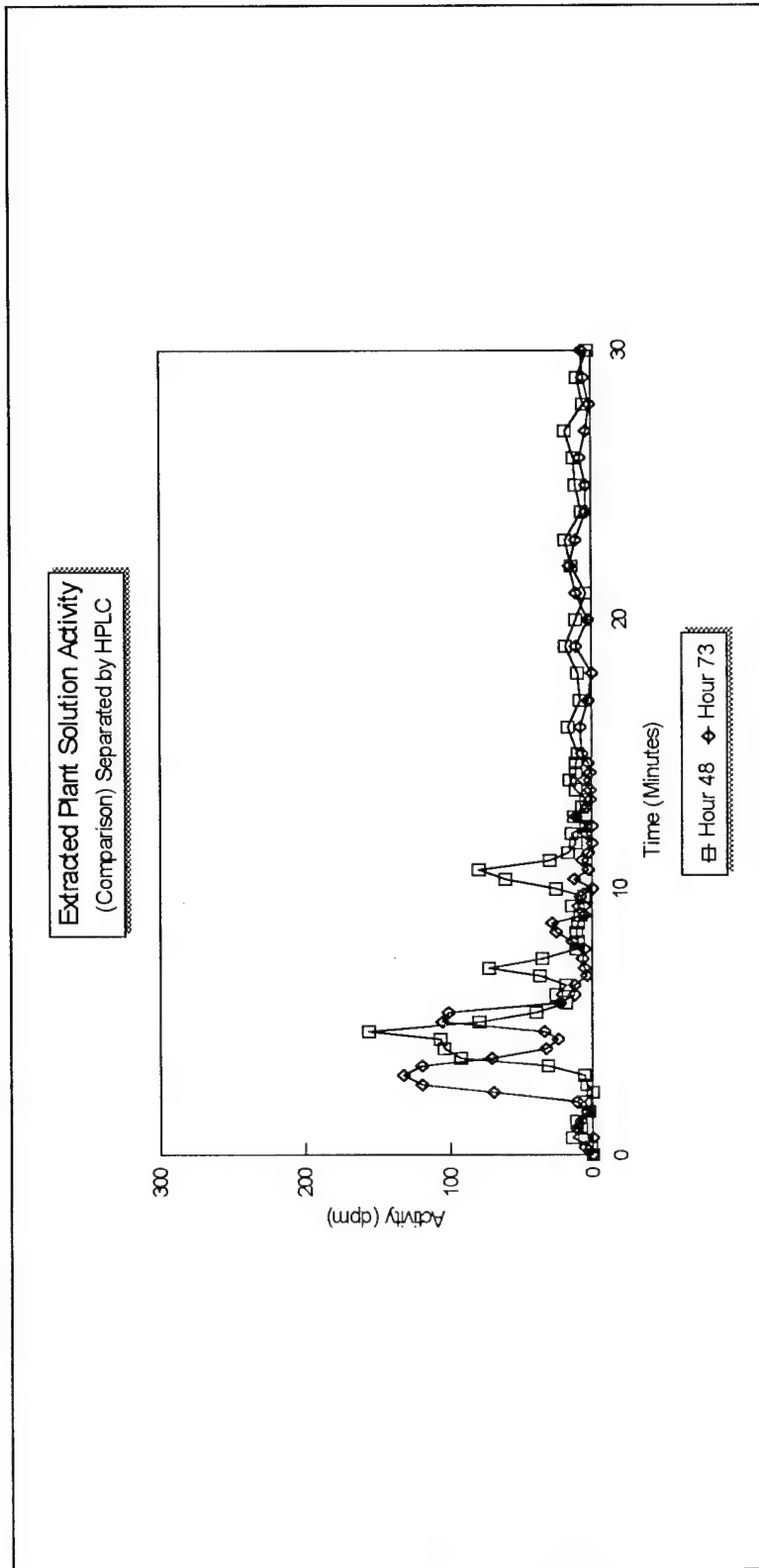


Figure D8
Extracted Plant Solution Activity Comparison, 48 and 73 hours.

Table D7
 Extracted Plant Solution ^{14}C Activity After Separation by
 HPLC
 Page 1 of 4

Time	^{14}C Extracted Plant Activity (dpm)				
(min)	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
0.0	0	0	0	0	0
0.3	1.34	0	0	1.36	5.32
0.7	0	0	0	14.02	0
1.0	4.84	0	1.4	8.38	12.37
1.3	3.98	0	0	11.43	8.42
1.7	2.25	0	0	3.51	4.45
2.0	3.99	5.77	6.25	6.61	12.41
2.3	3.56	26.03	78.75	0	70.51
2.7	47.11	63.46	116.68	4.38	119.83
3.0	75.69	77.6	104.73	5.75	132.15
3.3	79.66	118.07	136.96	31.24	118.85
3.7	115.49	64.77	72.66	92.32	71
4.0	32.62	33.54	27.36	104.62	33.17
4.3	26.89	15.48	14.16	106.86	24.66
4.7	9.28	5.37	20.31	156.38	33.88
5.0	0	11.5	8.41	79.64	105.52
5.3	15	38.77	7.11	39.54	100.69
5.7	9.29	20.74	8.45	19.33	22.49

Table D7
 Extracted Plant Solution ^{14}C Activity After Separation by
 HPLC

Page 2 of 4

Time	^{14}C Extracted Plant Activity (dpm)				
(min)	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
6.0	7.08	12.81	7.96	26.46	12.8
6.3	7.53	53.73	6.24	18.89	13.25
6.7	7.55	26.46	12.36	36.92	4.46
7.0	5.78	14.63	7.55	73.34	6.65
7.3	3.56	7.58	6.66	36.49	7.56
7.7	8.01	4.05	11.54	11.44	6.22
8.0	9.3	17.73	58.88	9.7	14.55
8.3	15.44	153.51	271.04	11.49	26.44
8.7	40.49	250.66	261.57	10.18	28.65
9.0	65.14	92.75	91.96	8.81	5.36
9.3	58.02	45.46	27.78	14.12	5.33
9.7	24.23	20.75	12.81	5.76	8.84
10.0	6.65	14.16	11.94	25.97	0
10.3	9.75	8.87	14.15	61.14	12.78
10.7	13.7	7.09	1.85	80.46	2.72
11.0	7.53	2.3	0.55	29.93	7.54
11.3	38.66	0.55	0	18.04	2.72
11.7	92.33	9.32	4.48	11.48	0

Table D7
 Extracted Plant Solution ^{14}C Activity After Separation by
 HPLC
 Page 3 of 4

Time	^{14}C Extracted Plant Activity (dpm)				
(min)	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
12.0	195.4	7.97	0	14.11	4.01
12.3	190.19	1.85	4.91	4.9	0.49
12.7	102.82	0	2.74	13.25	11.97
13.0	49.22	0	4.03	7.07	4.91
13.3	22.02	0	1.4	4.45	1.83
13.7	5.38	2.76	10.19	12.37	1.37
14.0	6.66	0	0	15.89	6.68
14.3	3.17	4.49	7.11	11.45	1.39
14.7	0.07	0.95	0.99	11.49	3.12
15	6.23	0	6.22	10.12	7.1
16	9.13	2.98	1.65	17.91	8.65
17	6	0.3	0	9.49	2.49
18	3.39	5.61	4.01	10.33	0.71
19	11.76	14.05	12.25	18.59	12.2
20	9.6	17.59	11.78	11.59	2.93
21	4.71	7.82	7.38	4.16	11.33
22	2.97	1.2	5.17	13.85	16.63
23	7.37	8.3	2.07	18.74	11.77

Table D7
Extracted Plant Solution ^{14}C Activity After Separation by
HPLC
Page 4 of 4

Time	^{14}C Extracted Plant Activity (dpm)				
(min)	3 Hour	12 Hour	24 Hour	48 Hour	73 Hour
26	3.37	2.52	1.18	13.03	9.56
27	0	5.18	2.94	18.76	4.69
24	4.3	14.91	13.57	7.26	4.67
25	6.49	6.97	2.06	11.68	5.11
28	2.48	0	3.38	5.96	1.6
29	0	0	6.47	9.93	6.47
30	3.85	4.75	4.69	3.3	7.32

Table D8
 TNT Reduction in a Water Due to Treatments of "Water Incubated With Ten Grams of
 Parrotfeather for Three Days" and "Ten Grams of Parrotfeather Plants"
 (Page 1 of 2)

Time (Hours)	TNT Concentration (ppm)					
	Beaker					
	1	2	3	4	5	6
0.0	60.0	60.0	60.0	60.0	60.0	60.0
0.3	63.7	35.2	46.1	36.9	60.3	56.7
0.5	60.8	37.1	37.5	39.4	56.2	47.6
1.0	56.0	51.3	46.4	38.5	55.9	47.3
1.5	58.0	42.1	35.1	47.1	57.6	33.2
2.0	37.6	59.3	45.8	30.1	37.7	33.2
2.5	58.6	56.6	42.8	34.5	42.8	34.6
3.0	43.2	58.2	43.0	26.5	35.0	39.5
3.5	45.3	45.5	36.2	35.1	48.4	31.7
4.0	48.5	47.5	51.3	39.5	59.6	20.7

Note: TNT concentration values for the water incubated with Parrotfeather for three days are shaded for emphasis.

Table D8
 TNT Reduction in a Water Due to Treatments of "Water Incubated With Ten Grams of
 Parrotfeather for Three Days" and "Ten Grams of Parrotfeather Plants"
 (Page 2 of 2)

Time (Hours)	TNT Concentration (ppm)					
	Beaker					
	1	2	3	4	5	6
5.0	37.8	56.1	30.2	26.6	44.9	26.2
6.0	33.2	57.2	31.4	21.1	38.5	25.6
7.0	59.7	41.4	22.1	23.5	61.0	30.2
22	26.4	39.7	5.6	9.8	55.1	5.4
25	18.7	55.0	4.9	7.0	54.3	4.3
31	32.8	36.9	2.2	4.1	58.3	2.2
45	32.0	41.8	0.0	0.1	37.9	0.1
54	44.0	44.0	6.0	ND		ND
93	43.0		6.0		53.0	

Note: TNT concentration values for the water incubated with Parrotfeather for three days are shaded for emphasis.

Table D9
TNT Concentration Versus Time Study Resulting from Three Differing Plant-to-Water Ratios
(Page 1 of 2)

Time (Hours)	TNT Concentration (ppm) Sorted By Plant-to-Water Ratios									
	One Gram (1:100)			Two Grams (1:50)			Ten Grams (1:10)			
0	119.0	119.0	119.0	119.0	119.0	119.0	119.0	119.0	119.0	119.0
0.5	97.0	85.1	88.6	98.7	109.5	77.9	61.3	74.0	32.4	
1	106.0	126.9	93.3	73.3	106.0	105.5	54.0	57.5	70.6	
1.5	119.6	85.9	82.6	93.2	97.3	93.7	47.6	58.4	69.6	
2	114.1	99.6	112.5	34.7	84.1	89.0	78.5	46.4	39.2	
2.5	93.8	106.8	103.8	104.1	69.2	94.6	77.5	74.3	43.1	
3	94.2	75.2	88.1	93.2	55.8	67.9	50.4	41.4	48.2	
3.5	65.0	75.0	82.0	52.0	54.0	71.0	28.0	24.0	21.0	
4	108.0	109.0	106.0	49.0	92.0	62.0	57.0	28.0	44.0	
5	108.0	109.0	106.0	49.0	92.0	62.0	57.0	28.0	44.0	

Table D9
TNT Concentration Versus Time Study Resulting from Three Differing Plant-to-Water Ratios
(Page 2 of 2)

Time (Hours)	TNT Concentration (ppm) Sorted By Plant-to-Water Ratios									
	One Gram (1:100)			Two Grams (1:50)			Ten Grams (1:10)			
6	62.0	77.0	54.0	54.0	76.0	61.0	22.0	35.0	24.0	
21	73.0	56.0	40.0		27.0	28.0	7.0	3.0	3.0	
24	57.0	92.0	82.0	21.0	39.0	40.0	1.0	1.0	2.0	
30	86.0	86.0	87.0	19.0	46.0	30.0	ND	ND	ND	
44	48.0	107.0	72.0	18.0	22.0	10.0	ND	ND	ND	
53	45.0	48.0	72.0	12.0	11.0	18.0	ND	ND	ND	
94		69.0	35.0		6.0	8.0	ND	ND	ND	
192	31.0	49.0	53.0	ND	ND	ND	ND	ND	ND	

Note: TNT concentration of ND = None Detected.

APPENDIX E

SPECTRAL PROFILES OF TNT AND TRANSFORMATION PRODUCTS
DETERMINED BY MECE

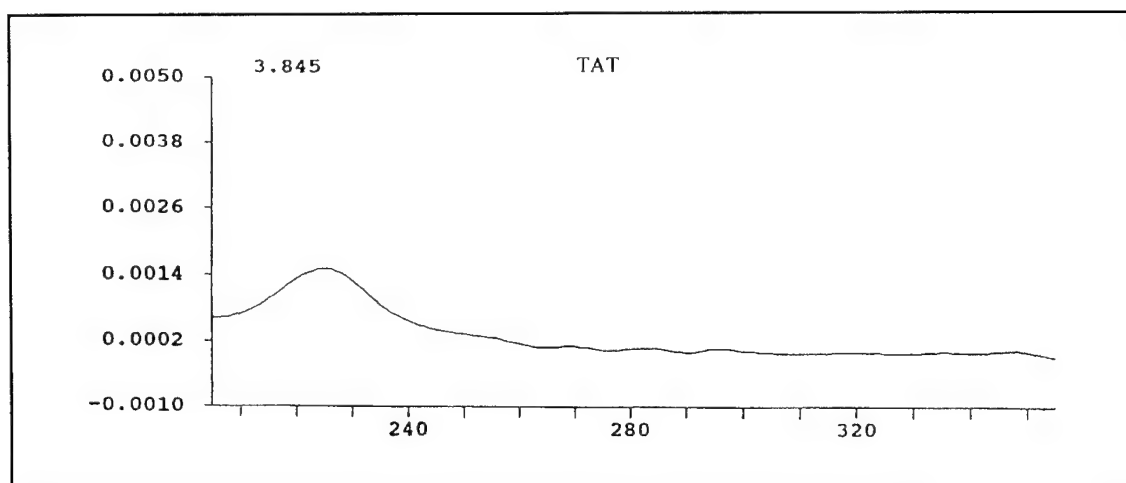


Figure E1
MECE Spectral Profile of a Triaminotoluene (TAT) Standard

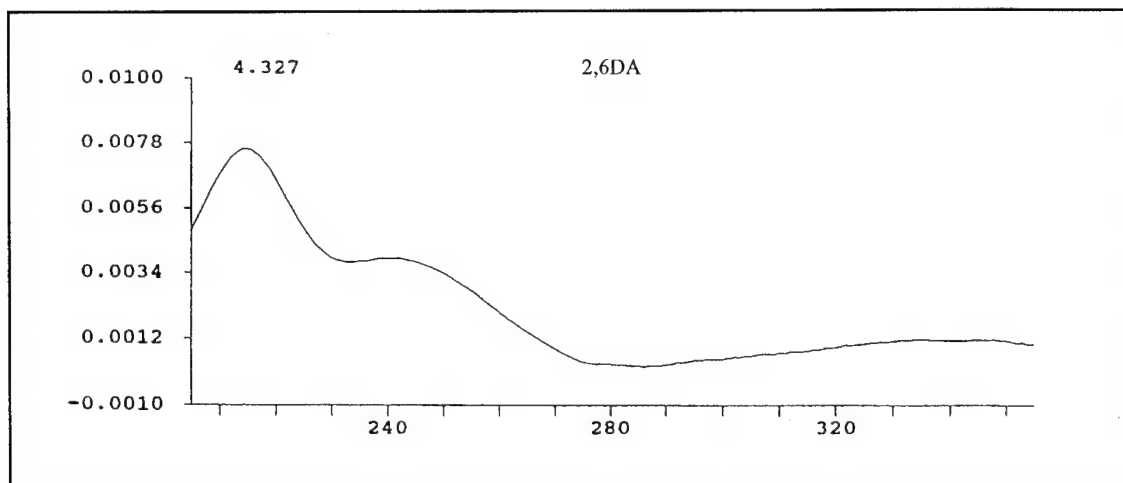


Figure E2
MECE Spectral Profile of a 2,6Diamino-4-Nitrotoluene (2,6DA)
Standard

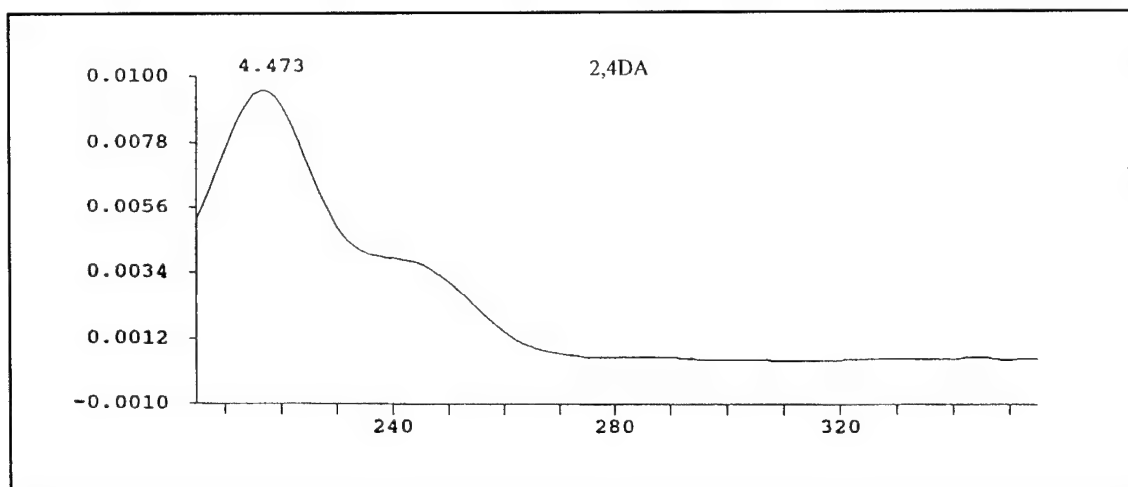


Figure E3
MECE Spectral Profile of a 2,4Diamino-6-Nitrotoluene (2,4DA)
Standard

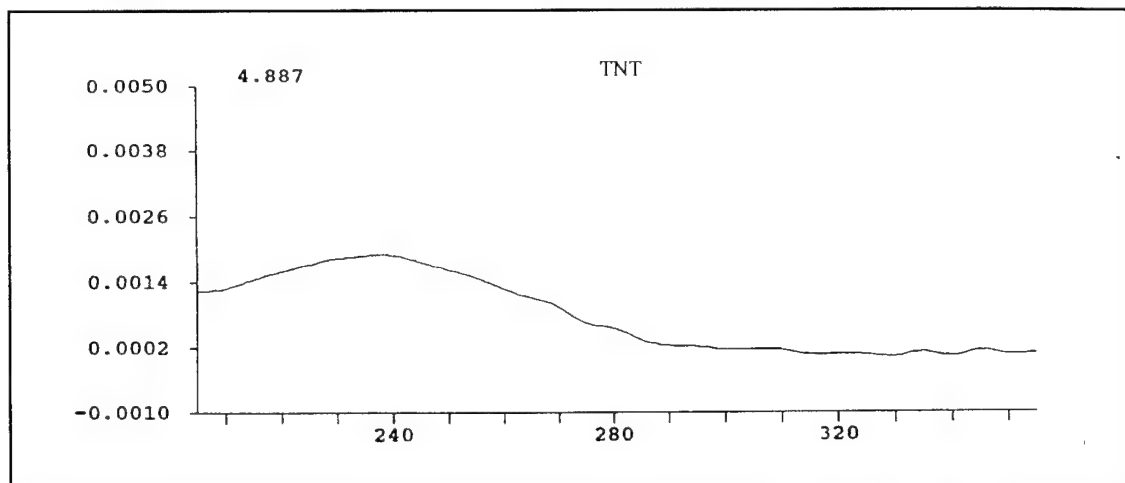


Figure E4
MECE Spectral Profile of a Trinitrotoluene (TNT) Standard

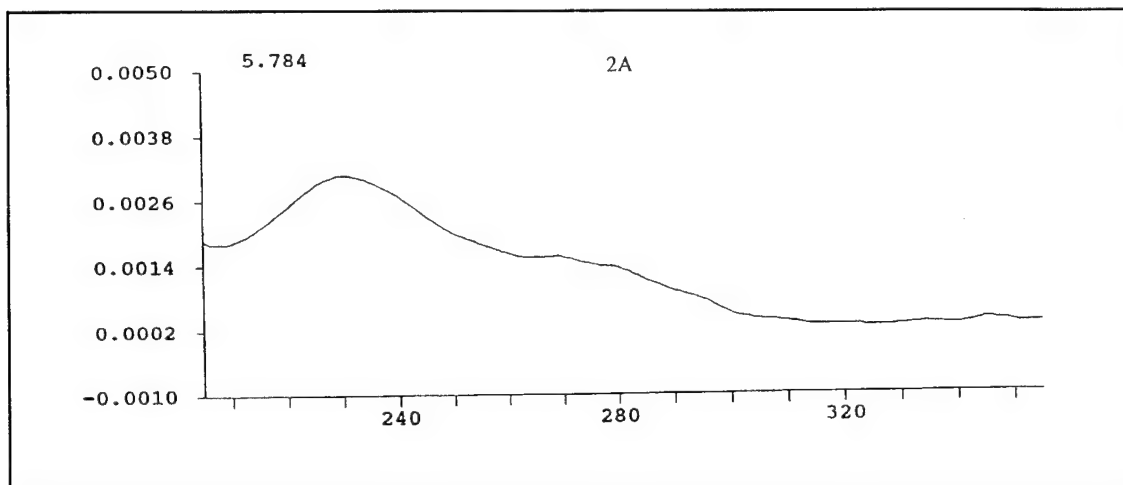


Figure E5
MECE Spectral Profile of a 2-Amino-4,6Dinitrotoluene (2A)
Standard

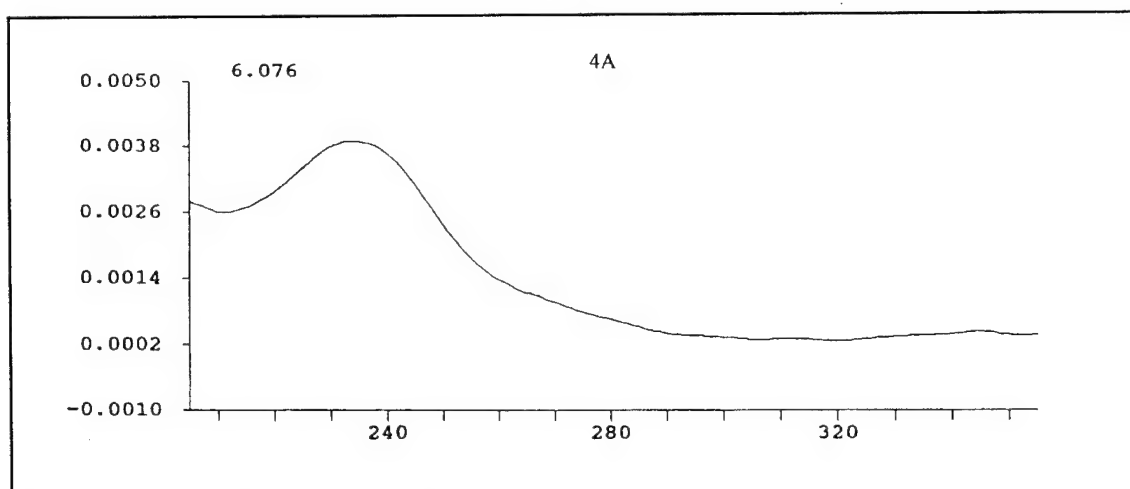


Figure E6
MECE Spectral Profile of a 4-Amino-2,6Dinitrotoluene (4A)
Standard

APPENDIX F
LISTING OF COMMON ABBREVIATIONS

COMMON ABBREVIATIONS

AAAP	Alabama Army Ammunition Plant
AAP	Army Ammunition Plant
ANOVA	Analysis of Variance
AU	Auburn University
DoD	Department of Defense
EPA	Environmental Protection Agency
ERL-A	EPA Research Lab, Athens, Georgia
GC/MS	Gas Chromatograph/Mass Spectrophotometry
HPLC	High Pressure Liquid Chromatography
ICAP	Inductively Coupled Argon Plasma
LAP	Load, Assemble, and Pack
MECE	Micellar Electrokinetic Capillary Electrophoresis
ND	None Detected
ppm	Parts per Million Parts
r^2	Least Squares Coefficient of Determination
SAS	Statistical Analysis System
TNT	2,4,6-Trinitrotoluene

APPENDIX G
LINEAR FIRST ORDER PLOTS

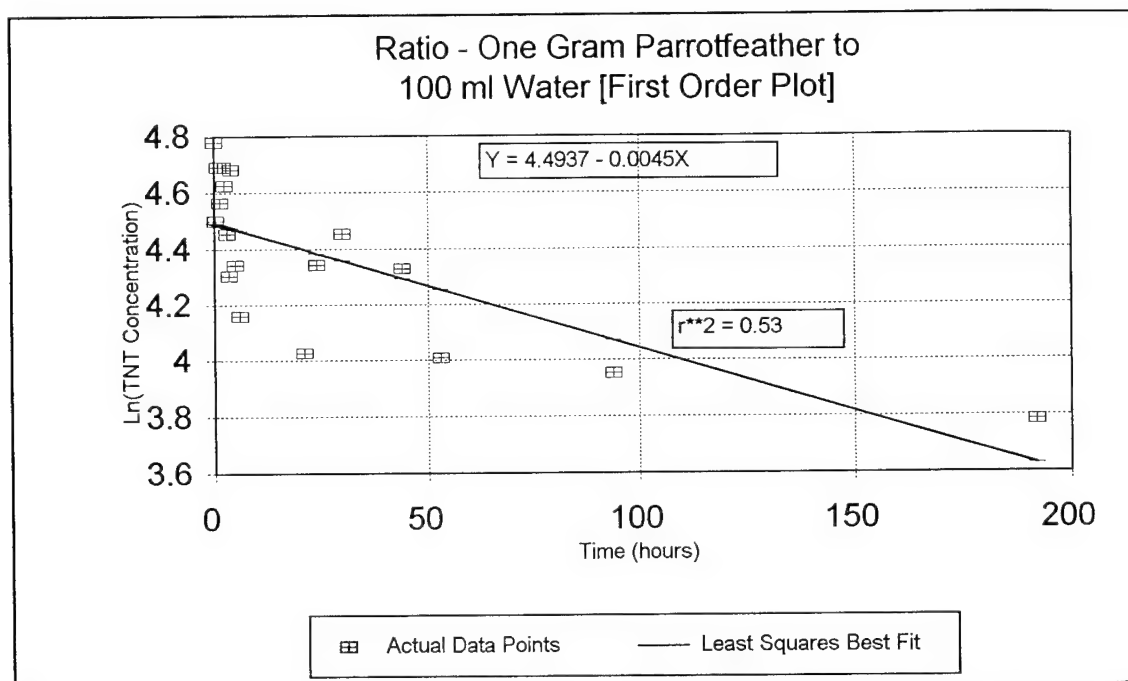


Figure G1

First Order Reaction Plot for the Resulting TNT
Concentration in 100 ml of TNT-Containing Water When
Incubated with One Gram of Parrotfeather

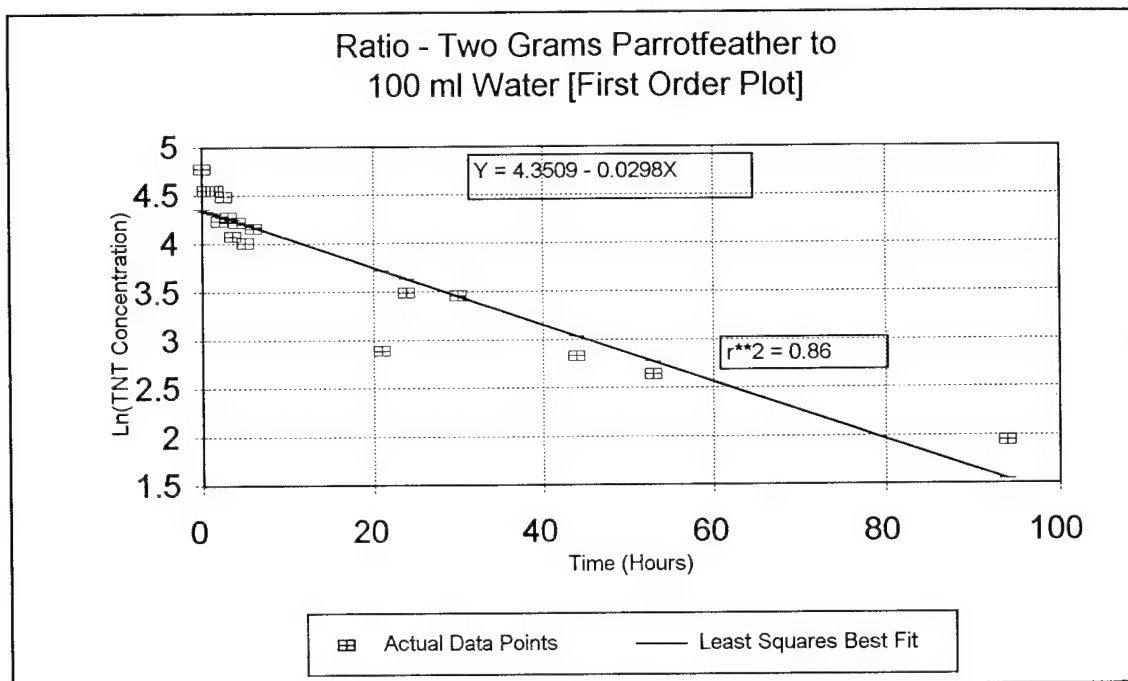


Figure G2

First Order Reaction Plot for the Resulting TNT
Concentration in 100 Ml of TNT-Containing Water When
Incubated with Two Grams of Parrotfeather

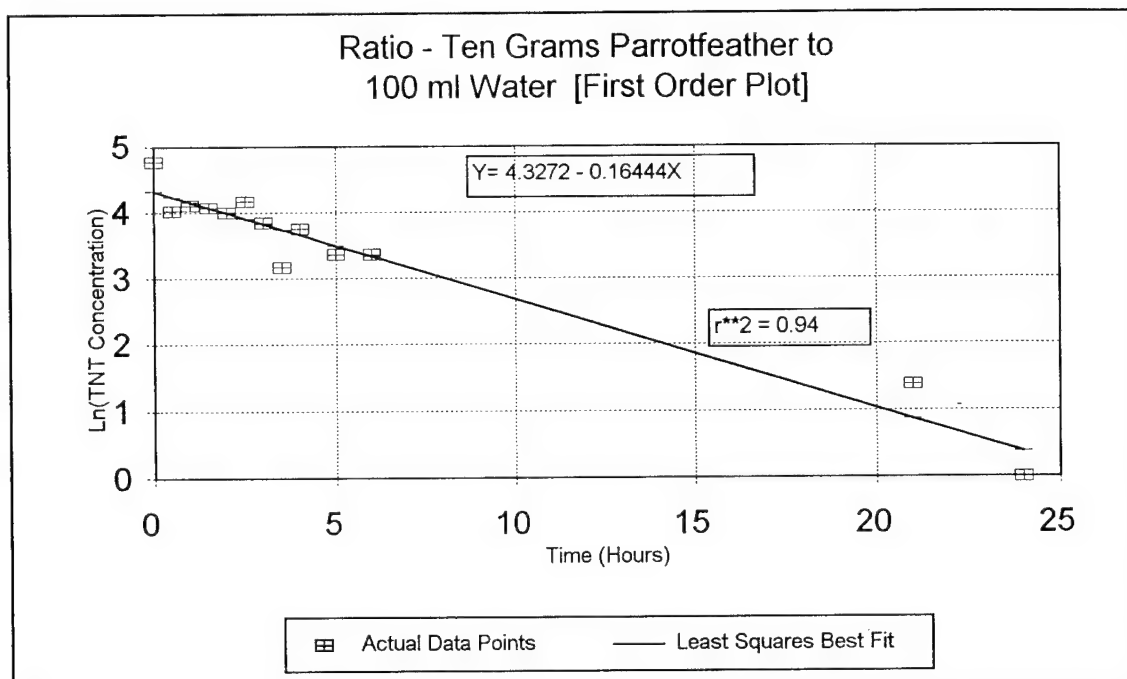


Figure G3

First Order Reaction Plot for the Resulting TNT
Concentration in 100 Ml of TNT-Containing Water When
Incubated with Ten Grams of Parrotfeather

APPENDIX H
FIELD EXPERIMENT PHOTOGRAPHS

Field Experiment Photograph Summary

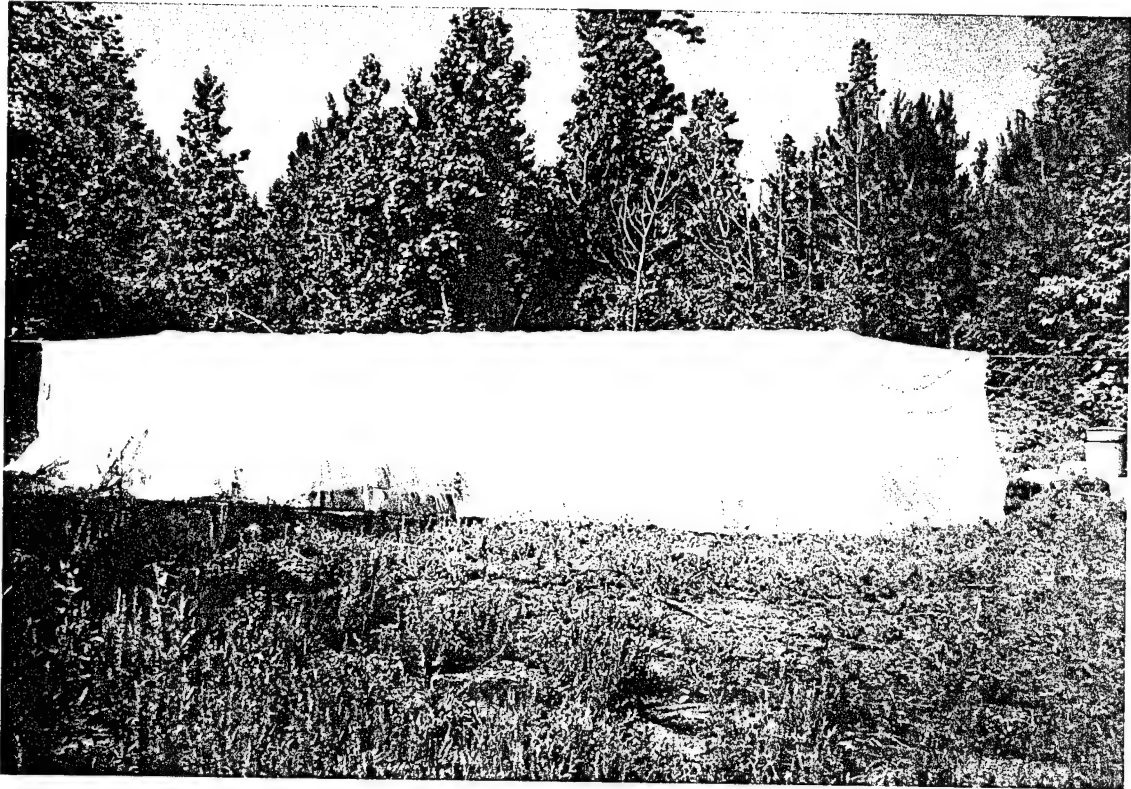
- H1: An exterior view of the greenhouse showing the ends covered for wind attenuation and heat retention during the winter months of the first field experiment.
- H2: A photograph of the AAAP beaver pond that supplied water and Parrotfeather for both field experiments.
- H3: A photograph of a freshly harvested Parrotfeather plant.
- H4: This photograph shows the second wash pool located just outside the greenhouse structure.
- H5: This is a view of the contaminated soil site.
- H6: This photograph shows a closeup view of the soil surface at the contamination site; TNT nuggets are clearly visible.

H7: This is a photograph of the TNT contaminated soil after it was prepared and stockpiled for the second field experiment.

H8: This photograph shows an interior view of the greenhouse as the soil is being measured and placed into the soil containers.

H9: This photograph shows the experimental containers just after the addition of Parrotfeather plants.

H10: This photograph shows the same view as the previous photograph but taken one week later.



Photograph H1



Photograph H2



Photograph H3



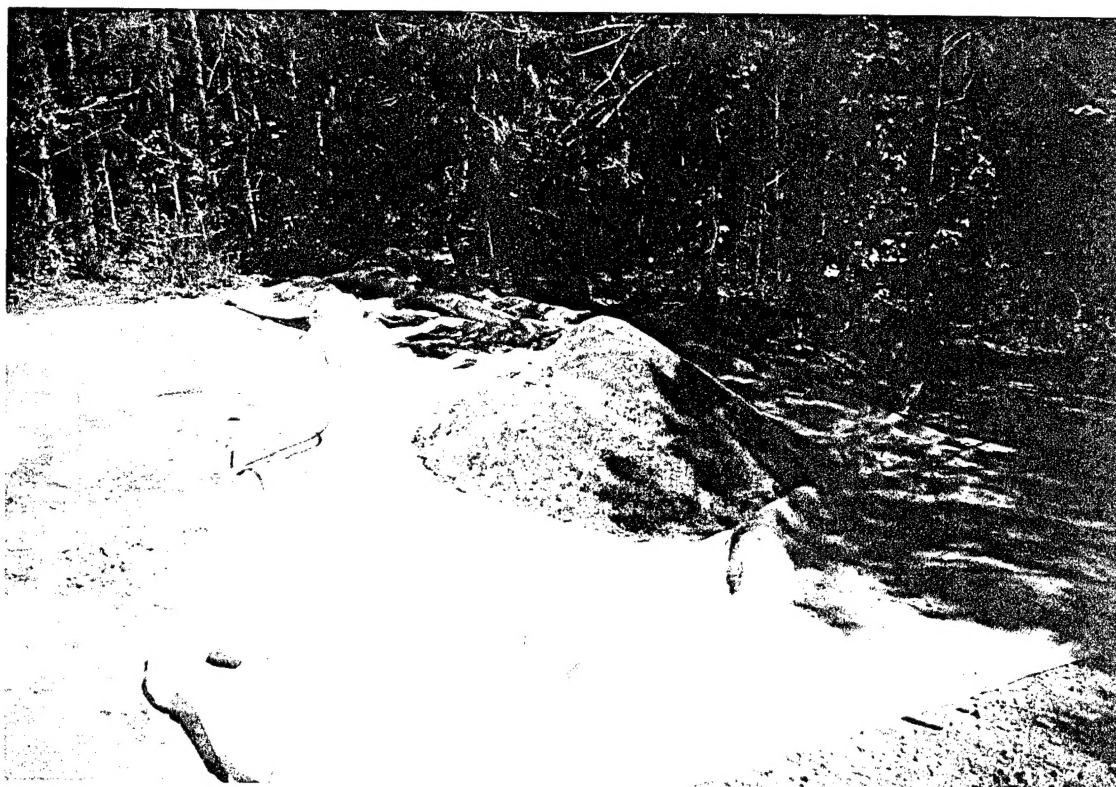
Photograph H4



Photograph H5



Photograph H6



Photograph H7



Photograph H8



Photograph H9



Photograph H10